Abstract. The present study aimed to determine the inhibitory effects of angiotensin II (AngII) on angiopoietin-like protein 2 (Angptl2) in rat primary cardiomyocytes, and to investigate the potential association between angiotensin II type 1 receptor (AT1R) and these effects. Cardiomyocytes were isolated from 3-day-old Wistar rats, and were cultured and identified. Subsequently, the expression levels of Angptl2 were detected following incubation with various concentrations of AngII for various durations using western blotting, reverse transcription-quantitative polymerase chain reaction, enzyme-linked immunosorbent assay and immunofluorescence. Finally, under the most appropriate conditions (100 nmol/l AngII, 24 h), the cardiomyocytes were divided into six groups: Normal, AngII, AngII + losartan, normal + losartan, AngII + PD123319 and normal + PD123319 groups, in order to investigate the possible function of AT1R in Angptl2 suppression. Losartan and PD123319 are antagonists of AT1R and angiotensin II type 2 receptor, respectively. The statistical significance of the results was analyzed using Student’s t-test or one-way analysis of variance. The results demonstrated that Angptl2 expression was evidently suppressed (P<0.05) following incubation with 100 nmol/l AngII for 24 h. Conversely, the expression levels of Angptl2 were significantly increased in the AngII + losartan group compared with the AngII group (P<0.01). However, no significant difference was detected between the AngII + PD123319, normal + losartan or normal + PD123319 groups and the normal group. The present in vitro study indicated that AngII was able to suppress Angptl2 expression, whereas losartan was able to significantly reverse this decrease by inhibiting AT1R.

Introduction

The renin-angiotensin-aldosterone system (RAAS) has an important role in homeostasis of the cardiovascular system (1). Angiotensin II (AngII) has an indispensable role in the RAAS, and is well recognized for its key function in cardiovascular and renal physiology (2). AngII not only mediates immediate physiological effects, including vasoconstriction and blood pressure regulation, but is also involved in inflammation, atherosclerosis, endothelial injury and heart failure (3). Heart failure is a complex clinical syndrome that lowers quality of life. It can be caused by hypertension, myocardial disease, coronary artery disease, or numerous other related heart disorders. RAAS inhibitors are widely used in the clinical therapy of heart failure. However, the exact mechanism underlying their effects remains to be fully elucidated. As a part of the RAAS, the effects of AngII are mediated by its interaction with angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) (4,5). The majority of the effects of AngII are mediated by AT1R, including extracellular matrix formation, increased vasopressin secretion, vasoconstriction, cardiac hypertrophy and cardiac contractility (6). AT1R is widespread in various organs and tissues, such as the liver, adrenal cortex and some areas of the brain. Vascular and myocardial tissues express more AngII compared with other tissues (7,8). Notably, a previous study revealed that angiotensin AT1 receptor-associated protein (ARAPI), which is also referred to as angiopoietin-like protein 2 (Angptl2), was suppressed in the kidney vasculature by AngII, which was mediated by AT1R (9). In this previous study, localization of Angptl2 was detected in the kidney of mice and humans, in order to confirm that Angptl2 expression can be regulated by AngII.

Angptl2 was initially cloned, expressed and characterized in 1999 (10). It is a member of the angiopoietin-like family, which contains eight members of glycoproteins. It is a secreted circulating protein with a molecular weight of 57 kDa, which consists of 493 amino acids (10-12). Members...
of the angiopoietin-like family possess a structure containing a typical N-terminal helical coiled-coil domain, a short linker peptide and a C-terminal globular fibrinogen-like domain, which is analogous to angiopoietins (10). Angptl2 has been detected in numerous mouse organs, with the highest expression levels found in the heart, aorta, kidney and adrenal gland (9). In addition, it has been reported that the distribution of Angptl2 is consistent with AT1R. The Angptl2 protein interacts with AT1R, and promotes AT1R translocation to the plasma membrane, facilitating increased AT1R surface expression in vitro (13). However, the expression levels of Angptl2 have not been detected in vitro following stimulation with AngII.

In the present study, a 48 h stimulation with 100 nmol/l AngII was initially conducted to observe alterations in Angptl2 expression; these conditions are considered the most appropriate for use in the kidney in vitro (9). The present study hypothesized that Angptl2 expression would be suppressed in rat cardiomyocytes following AngII treatment via AT1R. The most appropriate conditions (100 nmol/l AngII, 24 h) were identified in the present study, which were shown to significantly suppress Angptl2 expression. Several experiments were subsequently conducted to explore the role of AT1R in this process. Finally, a conclusion was drawn that the expression of Angptl2 may be suppressed by AngII via AT1R in rat cardiomyocytes.

Materials and methods

Isolation and culture of cardiomyocytes. Male, 3-day-old Wistar rats used in the present study were provided by the Animal Center of Shandong University (Jinan, China). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the ethics committee of Shandong University. Primary neonatal rat cardiomyocyte cultures were prepared as described in a previous study (14). Briefly, cardiomyocytes from Wistar rats were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Shanghai, China) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Beijing, China) at 37˚C in an atmosphere containing 5% CO2. The cardiomyocytes were incubated at a density of 5x10^6 cells/ml. Subsequent experiments were conducted on first generation primary cardiomyocytes.

Preparation of cultured cells. To illustrate the effects of AngII on Angptl2 expression, AngII (Sigma-Aldrich Chemie GmbH, Hamburg, Germany) was added to the culture medium in six-well plates containing cardiomyocytes (5x10^6 cells/ml) at the following final concentrations: 0, 50, 100 and 200 nmol/l for 24 h. In addition, cells were incubated with 100 nmol/l AngII for 0, 6, 24 or 48 h. The culture medium from each condition was collected. Total protein was extracted from the rat cardiomyocytes using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Beijing, China), which contained 0.1 M phenylmethylsulfonyl fluoride. After washing with phosphate-buffered saline (PBS), grinding, lysis and centrifugation (at 4˚C, 13,000 x g, 15 min), the supernatant was collected. Protein concentrations were measured using the bicinchoninic acid assay (Protein Assay kit; Beyotime Institute of Biotechnology), in which bovine serum albumin was used as a standard. After adding 5X loading buffer [sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Loading Buffer/Lane Markers; Beyotime Institute of Biotechnology], the mixture was boiled in water at 100˚C for 8 min, and was stored at -80˚C.

Immunofluorescence. α-Smooth muscle actin (α-SMA) was used as a marker to identify cardiomyocytes. The extracted cells were identified as cardiomyocytes by observing cellular morphology using immunofluorescent staining (14-16). Briefly, the cells were rinsed with PBS and were fixed with 4% paraformaldehyde for 15 min. Subsequently, they were permeabilized with 0.3% Triton X-100 for 20 min. Nonspecific antibody binding sites were blocked by incubating the cells in normal goat serum (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) for 30 min. The cells were then incubated with α-SMA (1:200; cat. no. 6487; Cell Signaling Technology, Inc., Danvers, MA, USA) and Angptl2 antibodies (1:400; cat. no. sc-292811; Santa Cruz Biotechnology, Inc.) overnight in a humidified incubator at 4˚C. The following day cells were incubated with Alexa Fluor 488 goat anti-rabbit immunoglobulin G secondary antibody (cat. no. A-11034; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 35 min at 37˚C and were washed three times with PBS. After the final wash, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 5 min. Images were acquired under an inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the rat cardio-myocytes following various treatment conditions using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration was determined using the Thermo Scientific Nano Drop 2000/2000c Spectrometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). cDNA was obtained by RT using Takara PrimeScript RT Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's specifications. qPCR was then performed to detect Angptl2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using the Applied Biosystems 7300 fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The mRNA expression levels of Angptl2 were normalized to GAPDH mRNA expression levels. qPCR amplification was carried out in a total volume of 20 µl, containing 10 ml SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.), 0.4 ml each of forward and reverse primers, 6.8 ml PCR-grade sterile water and 2 ml DNA templates.

The oligonucleotide primers used were as follows: Rat Angptl2, forward 5'-gaagcatagaacctgcctgc-3', reverse 5'-ctcgaactaagcagtctcag-3'; and rat GAPDH, forward 5'-gaagagagagcctctgc-3', and reverse 5'-tgtagaggtgatcagtc-3'. The cycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, 40 cycles at 95˚C for 5 sec, 60˚C for 34 sec and 95˚C for 15 sec, followed by 60˚C for 1 min and 95˚C for 15 sec. The ΔCq
values were obtained by subtracting the quantification cycle (Cq) value of GAPDH from the Cq value of Angptl2. The ΔCq values of each treatment group were further compared with those of the controls (ΔΔCq) to obtain relative quantification of the target gene expression (17).

SDS-PAGE and western blotting. Protein samples (100 μg) extracted from rat cardiomyocytes were separated by 10% SDS-PAGE and were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk at room temperature for 1 h. The membranes were then immunoblotted with Angptl2 (1:400; cat. no. sc-292811; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and GAPDH antibodies (1:10,000; cat. no. 60004-1-lg; Wuhan Sanying Biotechnology, Wuhan, China) at 4°C overnight, followed by goat anti-rabbit (1:3,000; cat. no. LK2001; Sanjian, Tianjin, China) and rabbit anti-mouse (1:5,000; cat. no. GTX29710; GeneFx, Inc., Irvine, CA, USA) secondary antibodies for 1 h. Subsequently, the images were detected using Luminol Reagent (EMD Millipore) and FluorChem E (ProteinSimple, San Jose, CA, USA). Analysis of western blotting was conducted using ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MD, USA).

Regulation of Angptl2 expression using AT1R and AT2R antagonists. To further explore the role of AT1R in Angptl2 expression regulation, additional experiments were conducted. AT1R antagonist (losartan; 10^{-4} mol/l; Sigma-Aldrich, St. Louis, MO, USA) and AT2R antagonist (PD123319; 10^{-4} mol/l; Abcam, Cambridge, UK) were added to the cells 45 min prior to treatment with AngII (100 nmol/l). After 24 h, protein, RNA and culture media were extracted. The normal plus losartan/PD123319 groups were used as negative controls.

Enzyme-linked immunosorbent assay (ELISA). The culture medium was harvested from the culture plates of various treatment groups. Angptl2 expression was measured using an Angptl2 detection kit (Human ANGPTL2 ELISA kit; R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer’s protocol. Angptl2 levels were calculated according to a standard curve and were corrected using an blank well.

Statistical analysis. Student’s t test was used to analyze differences between two groups. One-way analysis of variance with Bonferroni’s tests was used to analyze more than two groups. All analyses were performed using GraphPad Prism v 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA). All the experimental data are presented as the mean ± standard deviation and the mean values were calculated according to at least three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of cardiomyocytes. Primary cardiomyocytes were isolated from 3-day-old rats. The cellular morphology was observed under a microscope. Cardiomyocytes were shown to grow in a group, which adhered to the bottom of the dish (black arrow, Fig. 1A). In addition, the cardiomyocytes were detected to produce a certain beating rhythm under the microscope. The identification of cardiomyocytes was also verified by detecting α-SMA expression, which was chosen as a label of cardiomyocytes. Following incubation with α-SMA antibodies, the cells were stained with a secondary antibody, which was equipped with fluorescence (green), and DNA was stained with DAPI (blue). Fibroblasts cultured with cardiomyocytes exhibited negative staining for α-SMA. Images were captured at a magnification of x200 (Fig. 1B). Stained cells were counted under a microscope in four fields. The purity of cardiomyocytes in the isolated primary cells was ~94%.

Effects of various AngII concentrations on cardiomyocytes. To explore the effects of AngII on cardiomyocytes, the cells were incubated with various concentrations of AngII (0, 50, 100 or 200 nmol/l) for 24 h, or were incubated with 100 nmol/l AngII for various durations (0, 6, 24 or 48 h). As shown in Fig. 2A, the mRNA expression levels of Angptl2 were initially quantified in the cardiomyocyte samples, which were divided into two groups: The normal group and the cardiomyocyte group, which was stimulated with 100 nmol/l AngII for 48 h. The expression levels of Angptl2 were measured by RT-qPCR and were normalized to GAPDH. Treatment with 100 nmol/l AngII resulted in a decrease in Angptl2 mRNA expression (0.60±0.061) in rat cardiomyocytes compared with in the normal group (1.069±0.047; P<0.05; Fig. 2A). As shown in Fig. 2B and C, the protein expression levels of Angptl2 were measured following incubation for various durations with various AngII concentrations, respectively. The results demonstrated that following incubation with 100 nmol/l AngII for 24 h, the protein expression levels of Angptl2 (0.59±0.044) were more obviously decreased than under any of the other conditions, as compared with the normal group (1.78±0.029; P<0.05; Fig. 2B and C). An ELISA was also conducted to detect Angptl2 expression, in order to verify this trend. Culture media samples were used, which were collected from cells treated under the same conditions as for western blotting (Fig. 2D and E). The results of the ELISA were consistent with those of western blotting. Immunofluorescence was used to further verify the trend; DNA was stained with DAPI (blue) and Angptl2 was stained with green fluorescence (Fig. 2F). Blue and green fluorescence were assessed simultaneously under a magnification of x400. Representative images were captured under identical exposure parameters. ImageJ software was used to analyze the fluorescence (Fig. 2G). The expression levels of Angptl2 were markedly decreased following treatment with 100 nmol/l AngII for 24 h.

Role of AT1R and AT2R antagonists. Additional experiments were conducted to determine the possible role AT1R had with regards to this phenomenon. Following treatment with losartan (10^{-4} mol/l) and PD123319 (10^{-5} mol/l), the alterations in Angptl2 expression were detected in cardiomyocytes using western blotting, ELISA, RT-qPCR and immunofluorescence. Analysis of western blotting revealed that the protein expression levels of Angptl2 were significantly decreased in the AngII group (0.17±0.012) compared with in the normal group (0.52±0.018; P<0.05;...
Conversely, the AngII + losartan group exhibited increased Angptl2 expression (0.55±0.016) compared with the AngII group (P<0.05; Fig. 3A). Angptl2 expression in the normal + losartan group (0.61±0.0091) was similar to that in the normal group (P>0.05; Fig. 3A). The Angptl2 expression level in each group is shown in Fig. 3B. The expression level of Angptl2 in the AngII group was decreased significantly compared with the normal group (P<0.05), whereas the AngII + PD123319 group exhibited no difference compared with the AngII group. Additionally, the normal + PD123319 exhibited no significant difference compared with the normal group. RT-qPCR was also
performed to detect Angptl2 expression, and the results are displayed in Fig. 3C. Furthermore, the secretion of Angptl2 was detected using an ELISA. The ELISA results were consistent with those of western blotting (Fig. 3D). Immunofluorescence was performed concurrently and the results are presented in Fig. 3E. DNA was stained with DAPI (blue), and Angptl2 was stained using an antibody equipped with fluorescence (green). Representative images were captured under identical exposure parameters, and ImageJ was used to analyze fluorescence intensity (Fig. 3F). The RT-PCR
and immunofluorescence results were in accordance with the western blot analysis. These data suggest that Angptl2 is suppressed by AngII (100 nmol/l, 24 h), and this effect may be reversed by losartan (AT1R antagonist) but not by PD123319 (AT2R antagonist).

Discussion

Cardiac hypertrophy is an adaptive response that occurs in physical and pathological conditions, which ultimately results in heart failure. Pathological cardiac hypertrophy, which is caused by hypertension, myocardial infarction, cardiomyopathy or structural heart diseases, is associated with cardiac structural remodeling (18). Higher plasma levels of AngII have been detected in the heart of patients with cardiac hypertrophy and heart failure (19). AngII is involved in the RAAS and has vital roles in several diseases; the majority of effects of AngII are mediated by AT1R. Angptl2 is a member of the Angptl family, which has a vital role in AT1R recycling (20). Furthermore, Huang et al (21) revealed that Angptl2 serum levels in patients with heart failure are independently associated with heart failure. Therefore, Angptl2 may be related to cardiac hypertrophy; however, there is currently no evidence to verify its relationship with heart failure.

The results of the present study suggested that Angptl2 is suppressed by AngII in cardiomyocytes in vitro. The study speculated that AT1R may be the key factor mediating the effects of AngII in this process. To test this assumption an antagonist of AT1R was used, and an antagonist of AT2R was used as a negative control. The normal + losartan/PD123319 groups were also the negative control groups to testify the conclusion of this study. Analysis of the experimental data supported the aforementioned hypothesis.

Angptl2 is well recognized in several chronic diseases, including atherosclerosis, diabetes, metabolic disorders and cancer, due to its harmful proinflammatory properties (22). Angptl2 has been reported to act as a key inflammatory factor in activating adhesion molecule expression, thus increasing circulating cholesterol levels and accelerating the development of atherosclerotic plaques in dyslipidemic and pre-atherosclerotic mice (23). Angptl2 expression may increase following stimulation with AngII for 24 h. Undoubtedly, numerous experiments and further clinical proof are required to support this speculation in future studies.

Another possibility is that, as time prolongs and concentration increases, AT1R may saturate, and Angptl2 would increase to improve the sensitivity of AT1R to AngII. Further studies will lead to this effect. Since AT1R, which is an indispensable component of the RAAS, has a central role in several pathophysiological processes, Angptl2 may be involved in the process of cardiovascular diseases and function. The results of the present study may be used to propose a novel therapeutic method for the treatment of cardiovascular disease by decreasing Angptl2. Undoubtedly, numerous aspects of the present study require verification. The concentration range of AngII that suppressed Angptl2 in cardiomyocytes is not definite, and the explicit mechanism underlying this phenomenon is not certain. AngII is able to suppress Angptl2 in cardiomyocytes, and its effects on cardiomyocytes require further research. In addition, although Angptl2 and ARAP1 are encoded by the same gene, this does not necessarily mean that the matching proteins are entirely identical. More extensive experiments and further clinical proof are required to support this speculation in future studies.
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