Angiotensin II prompts heart cell apoptosis via AT1 receptor-augmented phosphatase and tensin homolog and miR-320-3p functions to enhance suppression of the IGF1R-PI3K-AKT survival pathway

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INTRODUCTION

Hypertension represents the largest health risk factor in modern society because it is a severe public health problem owing to its associated morbidity and mortality worldwide. Due to increases in the global population and population aging, the number of hypertensive patients has continued to increase in recent decades [1,2]. In the human body, blood pressure (BP) is regulated by the renin–angiotensin–aldosterone system (RAAS), which also maintains salt and water homeostasis through this hormone system in human body. However, RAAS also has an important role in the pathogenesis of hypertension [3,4], as it can control BP via the octapeptide angiotensin II (Ang II) mediated by angiotensin-converting enzyme hydrolysis. Ang II is a biologically active peptide at the center of the RAAS in BP regulation [4,5]. Ang II binds to the AT1 receptor (AT1R) to cause smooth muscle cell contraction, generate systemic vasoconstriction, release aldosterone, and reabsorb sodium to increase renovascular resistance, decrease renal BP, and increased BP. Ang II can also decrease BP by stimulating the AT2 receptor. Ang II–AT2 receptor interaction leads to vasodilation, natriuresis, and antiproliferative actions to decrease BP [3]. However, an

Background: Hypertension is a severe public health risk factor worldwide. Elevated angiotensin II (Ang II) produced by the renin–angiotensin–aldosterone system can lead to hypertension and its complications.

Method: In this study, we addressed the cardiac-injury effects of Ang II and investigated the signaling mechanism induced by Ang II. Both H9c2 cardiomyoblast cells and neonatal rat cardiomyocytes were exposed to Ang II to observe hypertension-related cardiac apoptosis.

Results: The results of western blotting revealed that Ang II significantly attenuated the IGF1R-PI3K-AKT pathway via the Ang II-AT1 receptor axis and phosphatase and tensin homolog expression. Furthermore, real-time PCR showed that Ang II also activated miR-320-3p transcription to repress the PI3K-Akt pathway. In the heart tissue of spontaneously hypertensive rats, activation of the IGF1R survival pathway was also reduced compared with that in Wistar-Kyoto rats, especially in aged spontaneously hypertensive rats.

Conclusion: Hence, we speculate that the Ang II-AT1 receptor axis induces both phosphatase and tensin homolog and miR-320-3p expression to downregulate the IGF1R-PI3K-AKT survival pathway and cause cell apoptosis in the heart.

Keywords: angiotensin II, heart damage, hypertension, miR-320, phosphatase and tensin homolog, renin–angiotensin–aldosterone system

Abbreviations: Ang II, angiotensin II; AT1R, AT1 receptor; BP, blood pressure; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGFI, insulin growth factor 1; IGF1-IR, IGF1 receptor; RAAS, renin–angiotensin–aldosterone system; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog
abnormal high level of Ang II causes many heart diseases, including hypertrophic cardiomyopathy and heart failure. The AT1R is thought to mediate the major cardiovascular effects of Ang II. The Ang II-AT1R pathway induces cardiomyocyte apoptosis, cardiac hypertrophy, and heart remodeling [6,7]. It’s determined Ang II as a critical factor in heart function. Another important factor for heart is insulin growth factor I (IGF1), which is involved in survival, oxidative stress, apoptosis, and proliferation [8]. Some studies have shown a synergistic effect between Ang II and IGF1. For example, the interaction between Ang II and insulin/IGF1 is found to be synergistic, rather than additive, in stimulating ERK1/2 activation in H295R cells [9]. In our previous studies, we present that IGF1 deficiency and/or IGF1 receptor (IGF-1R) resistance induces apoptosis in cardiomyoblast cells and that apoptosis is synergistically augmented by Ang II [10]. However, the mechanism underlying the synergy between Ang II and IGF1 is not clearly understood.

MicroRNAs are a class of small, endogenous, single-stranded, non-coding RNAs that maintain multiple biological processes in the cell [11]. Previous research has shown that miR-320-3p is an important miRNA that controls cell apoptosis. For example, miR-320-3p overexpression can cause apoptosis in hypoxic pulmonary arterial smooth muscle cells [12]. It also reduces cardiomyocyte survival after ischemia/reperfusion injury via Akt3 inhibition [13]. Some studies have shown that miR-320-3p might be involved in the IGF1R signaling pathway and could be involved in apoptosis and angiogenesis [14,15]. Phosphatase and tensin homolog (PTEN), a tumor suppressor gene, has biological functions in several different cells, including vascular smooth muscle cells, endothelial cells, and cardiomyocytes [16]. PTEN inhibits the phosphoinositide 3-kinase (PI3K) signaling pathway to negatively regulate cell growth, metabolism, and survival [17]. In heart, PTEN also acts as a negative regulator of several cardiovascular diseases. The level of PTEN is increased in the hearts of mice postmyocardial infarction, but a reduction of PTEN stimulates the PI3K/Akt/VEGF pathway to improve cardiac function and vascular remodeling in such animals [18]. Similarly, the inhibition of PTEN in human umbilical vein endothelial cells decreases oxidized LDL-induced apoptosis [19]. PTEN degradation further augments Wharton’s jelly-derived mesenchymal stem cell stability and protects cardiac function under hyperglycemic conditions [20]. Previous research has shown that miR-320 can cooperate with PTEN to repress tumor survival [21]. To date, however, there has been little discussion about the roles of both PTEN and miR-320 in the heart under hypertension stress.

It is known that hypertension represents a pathological state of the heart. However, the mechanism underlying the interactions among Ang II, IGF1R, miR-320, and PTEN in cardiomyocytes during hypertension remains elusive. To understand the biological interaction among Ang II, IGF1R, miR-320, and PTEN in cardiomyocytes under hypertension stress, we hypothesized that these factors would have an additive or synergistic effect on the heart induced by hypertension-associated cardiac damage in this study. The aim of this study was thus to address this.

### MATERIALS AND METHODS

**Materials**

Angiotensin II (Cat#: A9525), losartan (Cat#: SML3317), SF1670 (Cat#: SML6084), and most chemo reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless stated otherwise. The miR-320 mimic and inhibitor were purchased from Phalanx Biotech, Hsinchu, Taiwan.

**Cell culture and treatment**

Rat-derived H9c2 cardiomyoblasts were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and cultured in high-glucose DMEM (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, New York, USA), 2-mmol/l glutamine, 100-U/ml penicillin, 100-mg/ml streptomycin, and 1-mmol/l sodium pyruvate at 37 °C with 5% CO2 [22]. The cells were seeded and grown to 70–80% confluency before treatment.

**Neonatal rat cardiomyocyte isolation**

A neonatal Rat/Mouse cardiomyocyte isolation Kit (Cat#: nc-6031, Cellutron Life Technology, Baltimore, Maryland, USA) was used to isolate and culture neonatal rat cardiomyocytes, according to the manufacturer’s guidelines. Whole hearts from 1- or 2-day-old Sprague–Dawley rats (BioLASCO, Taipi, Taiwan) were collected, and cardiomyocytes were isolated with digestion solution at 37 °C. Collected cells were seeded in 10 cm plates at 37 °C for 2 h to reduce contamination by cardiac fibroblasts. All unattached cells were cultured on SureCoat pre-coated plates with NS medium with serum (Cat#:m-8031, Cellutron Life Technology) containing 100 U/ml penicillin, and 100 mg/ml streptomycin in a 5% CO2 incubator for 24 h. After 24 h, all media were changed to NW medium (Cat#:m-8032, Cellutron Life Technology) and cardiomyocyte cultures were ready for use in further experiments.

**Transfection assay**

The mimic and inhibitor of miR-320-3p were purchased from Phalanx Biotech (Hsinchu, Taiwan). H9c2 cardiomyoblasts or neonatal rat cardiomyocytes were transfected separately with the miR-320 mimic or miR-320 inhibitor using the jetPRIME transfection kit (Polyplus Transfection, Illkirch, France). After 6 h of transfection, Ang II was added to miR-transfected H9c2 cardiomyocytes or neonatal rat cardiomyocytes for 24 h.

**Animal heart tissue preparation**

The Animal Research Committee of China Medical University, Taichung, Taiwan approved all animal studies and all animal care (CMUICUC-2019-258). The study followed the Guide for the Care and Use of Laboratory Animals (8th ed.) [23]. Male Wistar-Kyoto rats (WKY) (three 5-month-old rats, three 18-month-old rats, and three 24-month-old rats) and age-matched male spontaneously hypertensive rats (SHR) (three 5-month-old rats, three 18-month-old rats, and three 24-old-aged rats) were purchased from BioLASCO Taiwan Co., Ltd. All rats were bred in an animal room (22°C and 50%
humidity with a 12-h light/12-h dark cycle) with drinking water and standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, Missouri, USA). WKY and SHR rats were allocated to the following groups: young (5-month-old) group, middle-aged (8-month-old) group, and aged (24-month-old) control group. All rats were sacrificed in a prefilled CO₂ chamber with 100% CO₂. The left ventricles were separated from the heart and aseptically collected for subsequent studies.

Protein extraction and Western blotting
The cell samples were prepared with RIPA buffer for 30 min on ice and centrifuged at 13,000 rpm for 60 min at 4 °C to collect the supernatants. The tissue extracts from each group were homogenized with RIPA buffer (100 mg tissue/1 ml radioimmunoprecipitation assay buffer (RIPA) buffer), placed on ice for 30 min, and centrifuged at 13,000 rpm for 40 min at 4 °C to collect the supernatants. The Bradford method (Bio-Rad Protein Assay; Bio-Rad, Hercules, California, USA) was used to determine the total protein concentration of each sample. The same amount (30 µg) of each protein sample was separated by SDS–PAGE at a constant voltage of 80 V. Electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Massachusetts, USA), phosphor-IGF1R, IGF1R, MAS, PI3K (Santa Cruz Biotechnology, Santa Cruz, California, USA), phospho-PI3K, PTEN (Cell Signaling Technology, Danvers, Massachusetts, USA), AT1R (Abcam, Cambridge, UK), and caspase 8 (Merck KGaA, Darmstadt, Germany) were diluted with antibody binding buffer and incubated with transferred PVDF membranes at room temperature for 1 h to avoid antibody non-specific binding. Primary antibodies, including those targeting β-actin, phospho-AktS473, Akt, caspase 3, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phospho-IGF1R, IGF1R, MAS, PI3K (Santa Cruz Biotechnology, Santa Cruz, California, USA), phospho-PI3K, PTEN (Cell Signaling Technology, Danvers, Massachusetts, USA), AT1R (Abcam, Cambridge, UK), and caspase 8 (Merck Millipore, Darmstadt, Germany) were diluted with antibody binding buffer and incubated with transferred PVDF membranes for approximately 12 h at 4 °C. The transferred PVDF membranes were then washed three times in TBST buffer for 10 min after specific antibody binding; the membranes were incubated with secondary antibodies (GE Healthcare, Chicago, Illinois, USA) at room temperature for 1 h. The membranes were then washed three times for 10 min with TBST buffer. Protein expression was visualized using an enhanced chemiluminescence western blotting luminal reagent and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan) [24–26].

RNA purification and quantitative real-time PCR analysis
RNA purification and quantitative real-time PCR analysis were performed according to previously described methods with slight modifications. Total RNA was collected using Total RNA Isolation Reagent – TRIzol Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and RNA samples (2 mg/ml) were converted to cDNA with reverse transcriptase using the Mir-X miRNA First-Strand Synthesis Kit (Takara Bio USA, Inc., San Jose, California, USA). cDNA (1 mg) was used with appropriate primers to perform real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.). The primer sequence for mir-320-3p and that of the U6 primer, as an internal control, were provided with the Mir-X miRNA Synthesis Kit. To normalize with the GAPDH housekeeping gene, cDNA was prepared with the iScript cDNA Synthesis kit following the kit’s protocol. Transcriptional levels of mir-320-3p, U6, and GAPDH expression were determined by SYBR green Connect Real-Time PCR Detection System (Bio-Rad). The expression levels of mir-320-3p were then normalized to U6 or GAPDH with a similar expression pattern. The inverse log of the DDCT was calculated [27–29].

TUNEL assay
The experimental methods of TUNEL assay were referred to the contents published by our laboratory with slight modification [30–32]. Cells were identified with a fluorescent microscope under an excitation wavelength in the 450–500 nm range and a detection wavelength in the 515–565 nm range (green). The positive signal was identified with an OLYMPUS BX53 microscope (Olympus Corporation, Shinjuku-ku, Tokyo, Japan).

Immunohistochemistry staining
When we sacrificed the animal and collected the heart, the hearts were fixed by formalin (neutral buffered, 10%). The sections were cut in 4 mm, deparaffinized by immersion in xylene, and rehydrated with gradient ethanol. The protocol of immunohistochemistry staining was referred to the contents published by our laboratory with slight modification [33,34]. Thereafter, the slides were identified with an OLYMPUS BX53 microscope (Olympus Corporation).

Statistical analysis
All experiments were performed at least three times. All results were quantified using ImageJ software and processed using Adobe Photoshop. Statistical analysis was conducted using SigmaPlot statistical software. Two groups were compared with Student’s t-test. Multiple comparisons of data were analyzed using a one-way analysis of variance, followed by Tukey’s test. Statistical significance was set at P less than 0.05.

RESULTS
Angiotensin II stimulates AT1 receptor expression to suppress the IGF1R survival pathway in H9c2 cardiomyoblasts
First, H9c2 cardiomyoblasts were utilized as a cell model to confirm that AT1R was upregulated by Ang II to repress the IGF1R signaling pathway. H9c2 cells were treated with Ang II, isoproterenol, and lipopolysaccharides, which are cardiotoxins, and only Ang II enhanced AT1R expression (Fig. 1a). The activation of IGF1R and Akt S473 phosphorylation was also reduced, but levels of the apoptosis markers cleaved caspase 8 and caspase 3 were increased (Fig. 1a). Next, different doses of Ang II and treatment periods were used to confirm that Ang II could repress the IGF1R pathway to induce heart cell apoptosis. As the concentration of Ang II was increased, AT1R expression also increased and IGF1R and Akt became less activated. The level of cleaved caspase 3 was also elevated following the increase in the Ang II concentration (Fig. 1b). The treatment periods of Ang II also affected AT1R and the...
downregulation of the IGF1R pathway. As time increased, Ang II also induced more AT1R to attenuate the phosphorylation of IGF1R and Akt S473 and upgraded cleaved caspase 8 and caspase 3 to activate cardiac cells apoptosis (Fig. 1c). These results suggest that AT1R expression can be enhanced by Ang II to suppress the IGF1R survival pathway and results in the apoptosis of H9c2 cardiomyoblasts. Further, Ang II-induced damage is dose-dependent and time-dependent.

Phosphatase and tensin homolog is upregulated by angiotensin II in H9c2 cardiomyoblasts

Next, we focused on PTEN as it is a negative regulator of the IGF1R-PI3K-Akt signaling pathway [17]. Different heart-damaging compounds were used to evaluate their effect on PTEN expression in H9c2 cardiomyoblasts. Results showed that Ang II increased PTEN expression (Fig. 2a). Ang II-induced PTEN expression was dose-dependent and time-dependent. High doses of Ang II and longer treatments also enhanced PTEN expression (Fig. 2b and c). To investigate the mechanism underlying the modulation of PTEN and AT1R mediated by Ang II-induced injury, we incubated Ang II-exposed H9c2 cells with a PTEN inhibitor (SF1670) or an AT1R inhibitor (losartan). From these results, we inferred that Ang II lost its ability to upregulate PTEN when losartan was added and the activity of Akt was rescued, which showed that Ang II could not perform its negative function when AT1R was unable to transmit its signal. Treatment with SF1670 also restored the Akt signaling that was downregulated by Ang II. When SF1670 and losartan were combined to treat Ang II-exposed H9c2 cells, the level of phospho-Akt was nearly recovered (Fig. 2d). These results indicate that Ang II upregulates PTEN by binding to AT1R and inhibits Akt phosphorylation to induce apoptosis. The inhibition of AT1R and/or PTEN can upregulate the activation of Akt and reduce cell apoptosis. Interestingly, losartan treatment also reduced the expression of AT1R and inhibited Ang II-AT1R signaling (Fig. 3). It suggests that PTEN inhibition may also affect Ang II-AT1R function.
Phosphatase and tensin homolog was upregulated by angiotensin II in H9c2 cardiomyoblasts. (a) Angiotensin II, isoproterenol, and lipopolysaccharides induced an increase in phosphatase and tensin homolog expression levels compared with control untreated H9c2 cardiomyoblasts. Angiotensin II: 100 nmol/l, isoproterenol: 50 nmol/l, and lipopolysaccharides: 2 μg/ml (b), angiotensin II treatment dose-dependently and (c), time-dependently upregulated phosphatase and tensin homolog expression levels. (d) SF1670 (phosphatase and tensin homolog Inhibitor) and losartan (AT1 receptor inhibitor) combination treatment with angiotensin II reversed angiotensin II-induced inhibition of Akt phosphorylation in H9c2 cardiomyoblasts. Angiotensin II: 200 nmol/l, SF1670: 2.5 μmol/l, and losartan: 1.5 μmol/l. The protein expression levels were normalized to internal control β-actin (a, b and c) or GAPDH (d). Results are expressed as protein fold change in treated groups compared with the control. Analysis of variance followed by the Tukey test was used to analyze the differences between the groups. Data are represented as mean ± SD from three independent experiments (ns: not significant, *P<0.05, **P<0.01, ***P<0.001).

Inhibition of the angiotensin II-AT1 receptor axis activated the IGF1R survival pathway to reduce apoptosis in angiotensin II-exposed H9c2 cardiomyoblasts. Losartan (an AT1 receptor inhibitor) reduced the expression levels of AT1 receptor and inhibited angiotensin II-AT1 receptor signaling. Downstream IGF1R was upregulated, which further enhanced the activity of Akt and inhibited apoptotic markers, including caspase 8 and caspase 3. The inhibition of the angiotensin II-AT1 receptor axis with losartan could be dose-dependent. The protein expression levels were normalized to internal control β-actin. The results are expressed as protein fold change in treated groups compared with the control. Analysis of variance followed by the Tukey test was used to analyze the differences between the groups. Data are represented as mean ± SD from three independent experiments (ns: not significant, *P<0.05, **P<0.01, ***P<0.001).
Moreover, downstream IGF1R was increased, which further enhanced the activity of Akt and inhibited the expression of apoptotic markers, including caspase 8 and caspase 3 (Fig. 3). The results demonstrate that the inhibition of Ang II and/or PTEN activates the IGF1R survival pathway to suppress Ang II-related apoptosis in the heart.

Angiotensin II upregulates miR-320-3p expression in H9c2 cardiomyoblasts

Previous literature mentions that miR-320-3p also affects cardiomyocyte survival via Akt3 inhibition [13]. Hence, we next examined the expression level of miR-320-3p in Ang II-exposed H9c2 cells via real-time PCR. As expected, miR-320-3p expression was increased in Ang II-exposed H9c2 cells (Fig. 4a, Supplementary Fig. 1A, http://links.lww.com/HJH/C68). With an increase in time with Ang II, the increase in miR-320-3p was even more significant (Fig. 4b, Supplementary Fig. 1B, http://links.lww.com/HJH/C68). However, the increased level of miR-320-3p was reduced by losartan (an AT1 receptor inhibitor) (Fig. 4c, Supplementary Fig. 1C, http://links.lww.com/HJH/C68). These results illustrate that Ang II binds to AT1R to stimulate the expression of miR-320-3p and suggest that miR-320-3p might play an important role in Ang II-induced effects on H9c2 cells.

Next, a mimic and inhibitor of miR-320-3p were employed to investigate the main function of miR-320-3p in Ang II-treated H9c2 cells. As the concentration of the miR-320 mimic was increased, both the total expression and phosphorylation of Akt decreased, showing that miR-320-3p downregulated AKT signaling (Fig. 5a). Upon cotreatment miR-320 mimic with Ang II, the results significantly showed that the levels of the signal molecules associated with PI3K and Akt were all downregulated (Fig. 5b, Supplementary Fig. 2A, http://links.lww.com/HJH/C68). In contrast, when the miR-320 inhibitor was cotreated with Ang II, it reversed the repression of the PI3K-AKT pathway caused by Ang II and also reduced the expression of cleaved caspase 3 (Fig. 5d, Supplementary Fig. 2B, http://links.lww.com/HJH/C68). We also used TUNEL assay to detect apoptotic cells in H9c2 cells. The image showed apoptosis was increased by Ang II and miR-320-3p mimic. On the contrary, the miR-320 inhibitor significantly reversed the response of Ang II-induced apoptosis (Fig. 6). We also isolated neonatal rat cardiomyocytes to confirm the role of Ang II-induced miR-320-3p in the heart. Similar results were observed in neonatal rat cardiomyocytes. These results presented that the miR-320 mimic attenuated the phosphorylation of AKT, but the miR-320 inhibitor rescued the activity of AKT in Ang II-treated neonatal rat cardiomyocytes (Fig. 7). All results indicate that Ang II-AT1R signaling upregulates miR-320-3p expression in Ang II-exposed heart cells and that miR-320-3p can directly repress cell survival signals, which has a synergistic effect with Ang II-AT1R signaling, causing heart damage.

The IGF1R-PI3K-AKT survival pathway is downregulated in hearts of a hypertensive animal model

To confirm our previous results, three different aged rat groups were used in this study. As expected, the activation
of IGF1R and AKT in the hearts of SHR rats was significantly lower than that in WKY rats. With increasing age, the IGF1R signaling pathway was associated with a greater reduction in activity in the hearts of hypertensive rats, especially in the oldest SHR rat group. Although all groups showed reduced expression of activated IGF1R and AKT with age, the IGF1R survival pathway in the WKY control group was always higher than that in the SHR group (Fig. 8a). The PTEN expression level was detected by immunohistochemistry staining, the similar result show SHR group was higher than WKY group. It could be support that PI3K-AKT pathway was reduced via PTEN in SHR group (Fig. 8b). These results provided evidence to confirm the reduction in the IGF1R survival pathway in the heart under hypertensive conditions. Interestingly, expression of the MAS1 receptor was also decreased in the hearts of SHR groups (Fig. 8a).

Previous studies have shown that the MAS1 receptor can bind Ang1-7 and physiologically oppose the actions of Ang II [35]. Hence, fewer MAS1 receptors were expressed in hypertensive hearts and might also lead to abnormal heart function.

**DISCUSSION**

Hypertension has been well recognized as one of the major chronic diseases leading to morbidity and mortality in the 21st century, and the number of patients presenting with this risk factor will continue to increase to more than 30% of the general population in the future [1,2]. Ang II from RAAS is the main regulator controlling BP in the human body, and elevated levels of Ang II can result in hypertension and its related cardiac complications and chronic heart failure [36].
In a previous study, it was confirmed that activation of the Ang II-AT1R axis in the heart causes cardiac hypertrophy and other related heart damage [6]. When human AT1R is overexpressed in the hearts of mice, the cardiac morphology exhibits massive hypertrophy, and the structures of atria and ventricles also become significantly larger [6]. Intriguingly, Mathieu et al. [37] reported that Ang II-AT1R-induced heart damage is associated with sex-specific differences. Female AT1R overexpressed mice have diminished Ca\(^{2+}\) dynamics, more heart dysfunction, and increased mortality compared with male AT1R overexpressed mice. It implies that hypertension induced via Ang II-AT1R axis might have varies by sex.

Activation of the IGF1R-PI3K-AKT signaling pathway is a cardiac-protective cascade. High-glucose-induced oxidative stress and HIF-1α transcriptional factor activation enhance IGFBP3 and inhibit the IGF1R-PI3K-AKT pathway to promote cardiomyocyte apoptosis [38]. However, the probiotic GMNL-263, oolong tea extract, or potato hydrolysate can trigger the IGF1R-PI3K-AKT survival pathway to protect heart functions [39–41]. In the current study, Ang II is not only found to bind AT1R to repress the IGF1R-PI3K-AKT survival pathway but also stimulated PTEN and miR-320-3p expression, resulting in a synergistic effect on suppression of the IGF1R-PI3K-AKT survival pathway. In the heart tissue of SHR rats, activation of the IGF1R survival pathway is also reduced compared with that in WKY rats, and the expression decreased with age.

PTEN, a tumor suppressor protein, is a multifunctional lipid phosphatase that negatively regulates the PI3K signaling cascades [17]. Generally, activation of the PI3K-AKT pathway in vascular smooth muscle cells, endothelial cells, cardiomyocytes, and cardiac fibroblasts improves survival and reduces apoptosis. However, loss of PTEN function can alter heart function. For example, loss of PTEN function can decrease pathological hypertrophy and improve resistance to heart failure to biomechanical stress [16]. Ravi et al. [42] show that dysregulation of PTEN is an essential role in the progression of pulmonary hypertension. Hyperglycemic stress induces PTEN and causes heart damage, but a reduction in PTEN enhances cell survival and suppresses diabetic cardiac damage [20]. However, one brief report presents that overexpression of PTEN in mice can reduce Ang II-induced vascular fibrosis and remodeling [43]. Cardiomyocyte-specific inactivation of PTEN leads to hypertrophy and a reduction in cardiac contractility [44]. Therefore, PTEN can play two opposing roles in heart. In our study, we show that PTEN plays a negative factor to reduced IGF1R-PI3K-AKT survival pathway. The Ang II-AT1R axis is found to upregulate PTEN to cause apoptosis; however, the inhibition of Ang II and/or PTEN activated the IGF1R-PI3K-AKT survival
FIGURE 7 The activation of AKT in angiotensin II-exposed neonatal rat cardiomyocytes was affected by miR-320-3p. (a) miR-320-3p overexpression, via a miR-320 mimic, significantly reduced the phosphorylation of AKT in angiotensin II-exposed neonatal rat cardiomyocytes. (b) miR-320-3p inhibition, via a miR-320 inhibitor, increased the phosphorylation of AKT in angiotensin II-exposed neonatal rat cardiomyocytes. The protein expression levels were normalized to internal control GAPDH. The results are expressed as protein fold change in treated groups compared with the control. Analysis of variance followed by the Tukey test was used to analyze the differences between the groups. Data are represented as mean ± SD from three independent experiments (ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001).

FIGURE 8 The IGF1R-PI3K-AKT survival pathway was reduced in the hearts of hypertensive rats. (a) All groups showed decreased expression of activated IGF1R and AKT with age, but the activation of IGF1R and AKT in the hearts of spontaneously hypertensive rats was significantly lower than that in Wistar-Kyoto rats. 5W: Wistar-Kyoto rats (5 months old); 5S: spontaneously hypertensive rats (5 months old); 8W: Wistar-Kyoto rats (8 months old); 8S: spontaneously hypertensive rats (8 months old); 24W: Wistar-Kyoto rats (24 months old); 24S: 24 months old. The protein expression levels were normalized to internal control β-actin. The results are expressed as protein fold change in treated groups compared with the 5-months-old Wistar-Kyoto rats. Analysis of variance followed by the Tukey test was used to analyze the differences between the groups. Data are represented as mean ± SD from three independent experiments (ns: not significant, *P < 0.05, **P < 0.01, ***P < 0.001). (b) The phosphatase and tensin homolog was detected by immunohistochemistry, the phosphatase and tensin homolog expression level was increased in spontaneously hypertensive rats (24 months old) compared with Wistar-Kyoto rats (24 months old). Representative images are shown. Scale bars: 50 μm.
Angiotensin II causes AT1 receptor pathways in heart tissues of SHR rats, activation of the IGF1R survival pathway was also reduced with age. Overall, as suggested by the current results in this study, the negative effect of Ang II on the heart is a synergistic effect, including AT1R, PTEN, and miR-320-3p (Fig. 9). The results of this study provide a more detailed biochemical mechanism to understand Ang II-induced cardiac injury.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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