

Roles of insulin-like growth factor II in cardiomyoblast apoptosis and in hypertensive rat heart with abdominal aorta ligation

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Lee, Shin-Da, Chun-Hsien Chu, Erh-Jung Huang, Min-Chi Lu, Jer-Yuh Liu, Chung-Jung Liu, Hsi-Hsien Hsu, James A. Lin, Wei-Wen Kuo, and Chih-Yang Huang. Roles of insulin-like growth factor II in cardiomyoblast apoptosis and in hypertensive rat heart with abdominal aorta ligation. *Am J Physiol Endocrinol Metab* 291: E306–E314, 2006; doi:10.1152/ajpendo.00127.2005.—Although IGF-II activating the IGF-II receptor signaling pathway has been found to stimulate cardiomyocyte hypertrophy, the role of IGF-II in cardiac cell apoptosis remains unclear. This study aimed to identify the roles of IGF-II and/or IGF-II receptors (IGF-II/IIR) in cardiomyoblast apoptosis and in hypertensive rat hearts with abdominal aorta ligation. Cultured rat heart-derived H9c2 cardiomyoblasts and excised hearts from Sprague-Dawley rats with 0- to 20-day complete abdominal aorta ligation, a model of ANG II elevation and hypertension, were used. IGF-II/IIR expression, caspase activity, DNA fragmentation, and apoptotic cells were measured by RT-PCR, Western blot, agarose gel electrophoresis, and TUNEL assay following various combinations of ANG II, IGF-II/IIR antibody, CsA (calcineurin inhibitor), SP-600125 (JNK inhibitor), SB-203580 (p38 inhibitor), U-0126 (MEK inhibitor), or Staurosporine (PKC inhibitor) in H9c2 cells. ANG II-induced DNA fragmentation and TUNEL-positive cells were blocked by IGF-II/IIR antibodies and antisense IGF-II, but not by IGF-II sense. IGF-II-induced apoptosis was blocked by IGF-IIR antibody and CsA. The increased gene expressions of IGF-II and -IIR induced by ANG II were reversed by U-0126 and Sp600125, respectively. Caspase 8 activities induced by ANG II were attenuated by U-0126, SP-600125, and CsA. DNA fragmentation induced by ANG II was totally blocked by SP-600125, and CsA and was attenuated by U-0126. In rats with 0- to 20-day complete abdominal aorta ligation, the increases in IGF-II/IIR levels in the left ventricle were accompanied by hypertension as well as increases in caspase 9 activities and TUNEL-positive cardiac myocytes. ANG II-induced apoptosis was reversed by IGF-II/IIR blockade and coexisted with increased transactivation of IGF-II and -IIR, which are mediated by ERK and JNK pathways, respectively, both of which further contributed to cardiomyoblast apoptosis via calcineurin signaling. The increased cardiac IGF-II, IGF-IIR, caspase 9, and cellular apoptosis were also found in hypertensive rats with abdominal aorta ligation.

angiotensin; apoptosis; growth factor; cardiomyocyte; signal transduction

Apoptosis, which is characterized by DNA fragmentation, is known to increase caspase activities and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)-posi-

tive cells and could potentially be important in many cardiac disorders (12, 15). Apoptosis in cardiac cells may be mediated via a variety of biological signaling pathways, including calcineurin, protein kinase C (PKC), c-Jun NH₂-terminal kinase (JNK), mitogen-activated protein kinase (MEK), p38 mitogen-activated protein kinase (p38), and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK-MAPK) pathways (23). An elevation in angiotensin II (ANG II) level is commonly observed in cardiovascular diseases, including hypertension, coronary artery disease, left ventricular hypertrophy, and heart failure (17). There is a growing body of evidence suggesting that ANG II functions as a regulator of apoptosis in cardiac cells (12). Most of the evidence indicates that the stimulation of ANG II in the heart is associated with an increased rate of myocardial apoptosis (9, 16). Although the mechanism of apoptosis enhanced by ANG II is still controversial due to a variety of possible pathways, the calcium-calcineurin pathway has been regarded as an important one for ANG II-induced apoptosis in cardiac cells (14, 19, 21). ANG II has been shown to activate calcineurin through G_{αq}/PLC signaling transduction. Calcineurin is mediated by permeability transition pore formation and activates the mitochondrial apoptotic pathway (21). The activations of JNK, ERK, and p38 pathways may be responsible for ANG II-induced cardiac cell hypertrophy (3), whereas the roles of ERK, JNK, PKC, and p38 in ANG II-induced cardiomyoblast apoptosis remain controversial.

Cardiac myocytes express relatively high levels of IGF-II after ischemia induced by occlusions and reperfusion of the left anterior descending coronary artery (20). In addition to binding with the IGF-II receptor (IGF-IIR), IGF-II can bind with the insulin receptor A subtype and IGF-IR (24). When IGF-II activates the IGF-IIR signaling pathway, IGF-II might activate calcineurin through G_i protein signaling transduction and stimulate cardiac cell hypertrophy (2, 18). To our knowledge, however, it is still unclear whether relatively high levels of IGF-II stimulate cardiomyocyte apoptosis. Calcium-calcineurin has been shown to dephosphorylate proapoptotic Bcl-2 family protein-Bad and to induce cytochrome *c* release from the mitochondria to the cytosol, which may further induce caspase activities and apoptosis (7). However, it is still un-

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known whether calcineurin is involved in the role of IGF-II in cardiomyocyte apoptosis. It is also unclear whether ANG II regulates IGF-II/IIR signalings and further activates calcium-calcineurin or other pathways in cardiac cell apoptosis.

Although an elevation in ANG II level and an increase in blood pressure have been reported in an animal model with a complete abdominal aorta ligation (1), we do not know whether IGF-II/IIR expressions and cardiac apoptotic activity would be increased in a similar context. In this study, we hypothesize that ANG II-induced cardiomyoblast apoptosis is regulated by IGF-II/IIR systems via the calcium-calcineurin pathway and that the cardiac IGF-II system and cardiac apoptosis are induced by complete abdominal aorta ligation.

ANG II-induced apoptosis has been found in cultured cardiomyoblast H9c2 cells and in hearts excised from a hypertensive rat model with abdominal aorta ligation. We found that the ANG II-induced apoptosis in cardiomyoblast H9c2 was attenuated by IGF-II or IGF-IIR blockade and that ANG II-induced apoptosis coexisted with upregulation of IGF-II and IGF-IIR. The upregulated IGF-II and IGF-IIR were mediated by the ERK and JNK pathways, respectively, both of which further contribute to cardiomyocyte apoptosis via calcineurin signaling. In addition, the increased IGF-II, IGF-IIR, caspase 9 activities, and cellular apoptosis were also found in the hearts excised from hypertensive rats with abdominal aorta ligation.

METHODS

Cell Culture

H9c2 cardiomyoblast cells were obtained from American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM pyruvate in humidified air (5% CO₂) at 37°C. H9c2 cells were cultured in serum-free medium for 12 h and then treated with or without ANG II (10⁻⁸ M; Sigma Chemical, St. Louis, MO), antisense IGF-II (14 µM), sense IGF-II (14 µM), IGF-II antibody (100 ng/ml; Neo Markers, Fremont, CA), or IGF-IIR antibody (100 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA). The incubation was continued for another 24 h, and then the cells were harvested and extracted for analysis.

Animal Preparation

Sixty-seven male Sprague-Dawley rats were purchased from the National Laboratory Animal Breeding and Research Center, Taipei, Taiwan. Ambient temperature was maintained at 25 ± 1°C, and the animals were kept on an artificial 12:12-h light-dark cycle from 7 AM to 7 PM. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International, Brentwood, MO) and water ad libitum. Under pentobarbital sodium (1 ml/kg) anesthesia, all animals were processed by either sham operation or complete ligation of the abdominal aorta between the two origins of the renal arteries. The rat with ligation of the abdominal aorta was a well-developed animal model with an elevation in ANG II level, systemic hypertension, and cardiac hypertrophy. Thus, after ligation on 0 day (sham, *n* = 8), 1 day (sham, *n* = 4 vs. ligation, *n* = 4), 2 days (sham, *n* = 5 vs. ligation, *n* = 5), 3 days (sham, *n* = 7 vs. ligation, *n* = 6), 5 days (sham, *n* = 3 vs. ligation, *n* = 3), 7 days (sham, *n* = 4 vs. ligation, *n* = 4), 10 days (sham, *n* = 4 vs. ligation, *n* = 4), and 20 days (sham, *n* = 3 vs. ligation, *n* = 3), systolic, diastolic, and mean arterial blood pressures were determined with a sphygmomanometer (29SSP; IITC/Life Science Instruments) in all rats. Then, these animals were killed, and the hearts were removed for the further analysis. All protocols were approved by the Institutional Animal Care and Use Committee of

Chung Shan Medical University and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Design and Synthesis of IGF-II Oligonucleotides and Transfection

The 15-mers of IGF-II antisense oligonucleotides and sense oligonucleotides used in this study target the translation initiation site of IGF-II mRNAs of the rats, and antisense IGF-II oligonucleotides were complementary to nucleotides (5'-ATG GGG ATC CCA GTG-3'). In addition, the IGF-II sense oligonucleotides were the same as nucleotides (5'-ATG GGG ATC CCA GTG-3') used as negative control. The sequences had no similarity to other mammalian genes. All antisense and sense oligonucleotides were synthesized as lyophilized powders and reconstituted in sterile, nuclease-free Tris-EDTA buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.4; MDBio, Taipei, Taiwan) and were stored at -20°C. Cells were grown to 80% confluence in 100-mm dishes. IGF-II oligonucleotides and Lipofectamine were separately diluted in serum-free DMEM to 200-µl volumes, mixed, and incubated at room temperature for 20 min. Cells were washed twice with PBS and overlaid with 5 ml of serum-free DMEM, to which the DNA lipid complexes were added. The cells were incubated for 6 h at 37°C, whose medium was replaced with DMEM-10% FBS, and harvested after 24 h.

TUNEL

After various treatments, H9c2 cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with phosphate-buffered saline (PBS), the samples were first incubated with phalloidin-rhodamine for 1 h and with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP (Roche Applied Science, Indianapolis, IN). In heart tissues, the 3-µm-thick paraffin sections were deparaffinized by immersion in xylene, rehydrated, and incubated in PBS with 2% H₂O₂ to inactivate endogenous peroxidases. Next, the sections were incubated with proteinase K (20 µg/ml), washed in PBS, and incubated with terminal deoxynucleotidyl transferase for 90 min and fluorescein isothiocyanate-dUTP for 30 min at 37°C using an apoptosis detection kit (Roche Applied Science). Then, the sections were stained with 4,6-diamidino-2-phenylindole to detect cell nucleus by UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope at this state, respectively, using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green). The number of TUNEL-positive cardiac myocytes was determined by counting 3 × 10⁵ cardiac myocytes. All morphometric measurements were performed by at least two individuals independently in a blinded manner.

DNA Fragmentation

H9c2 cells were lysed in 50 µl of lysis buffer (50 mM Tris·HCl, pH 7.4, 20 mM EDTA, 1% Igepal-630) followed by incubation with 1% SDS and 5 µg/µl RNase (Roche Molecular Biochemicals, Mannheim, Germany) for 2 h at 56°C and 2.5 g/µl proteinase K (Roche) for 2 h at 37°C, and only fragmented DNAs were extracted. DNAs were ethanol precipitated and finally resuspended in distilled water. The fragmented DNAs were electrophoretically fractionated on a 1.5% agarose gel and stained with ethidium bromide.

Inhibitors

H9c2 cells were treated with several inhibitors, including SB-203580 (p38 MAP kinase inhibitor; Promega), CsA (calcineurin inhibitor), U-0126 (MEK1 and MEK2 inhibitor; Promega), SP-600125 (JNK inhibitor; Promega), LY-294002 (PI 3-kinase inhibitor; Promega), and Staurosporine (PKC inhibitor). The final concentrations of inhibitors were 10 µM in SB-203580, 1 µM in CsA, 30

μM in U-0126, 20 μM in SP-600125, 10 μM in LY-294002, and 20 μM in Staurosporine.

Total RNA Extraction

Total RNA was extracted using the Ultraspec RNA isolation System (Biotex Laboratories, Houston, TX) according to directions supplied by the manufacturer. Respectively, H9c2 cells and cardiac tissues were thoroughly homogenized (1 ml Ultraspec reagent/100 mg tissue cell) with a homogenizer. The RNA precipitate was washed twice by gentle vortexing with 70% ethanol, collected by centrifugation at 12,000 g, dried under vacuum for 5–10 min, dissolved in 50–100 μl of diethylpyrocarbonate-treated water, and incubated for 10–15 min at 55–60°C.

Reverse Transcription and PCR Amplification

cDNA was prepared in a buffer containing 50 mM Tris·HCl, pH 8.5, 30 mM KCl, 8 mM MgCl, 1 mM dithiothreitol, 0.25 mM each dCTP, dGTP, dTTP, and dATP, 20 U of recombinant ribonuclease

inhibitor, 1 pg of random hexamers, 5 pg of total RNA, and 40 U of avian myeloblastosis virus reverse transcriptase in a volume of 20 pl. This mixture was incubated for 10 min at room temperature followed by 1 h at 42°C to initiate cDNA synthesis. This mixture was then used for amplification of specific cDNAs by PCR. The buffer for PCR contained 50 mM KCl, 10 mM Tris·HCl, pH 8.3, at 20°C, 0.2 mM each dCTP, dGTP, dTMP, and dATP, 0.5 pM oligonucleotide PCR primers, 2.5 U of *Taq* polymerase, and various MgCl concentrations in a final volume of 100 pl. Following the hot start (5 min at 95°C, 80°C hold), the samples were subjected to 35 cycles of 45 s at 95°C, 2 min at 52°C, and 45 s at 72°C. For the IGF-II, IGF-IIR, and GAPDH primers, the primer annealing temperature was 56°C. This was followed by a final extension step at 72°C for 10 min. All RNA samples used were demonstrated to have intact 18S and 28S RNA bands on ethidium bromide-stained formaldehyde-agarose gels. Primers were as follows: rat IGF-II forward primer CTTGTTGACACGCTTCAGT, reverse primer GTTCACTGATGGTTGCTGGA; rat IGF-IIR forward primer GCAAGGGCATAAAGGTGAA, reverse primer TGTAAGT-

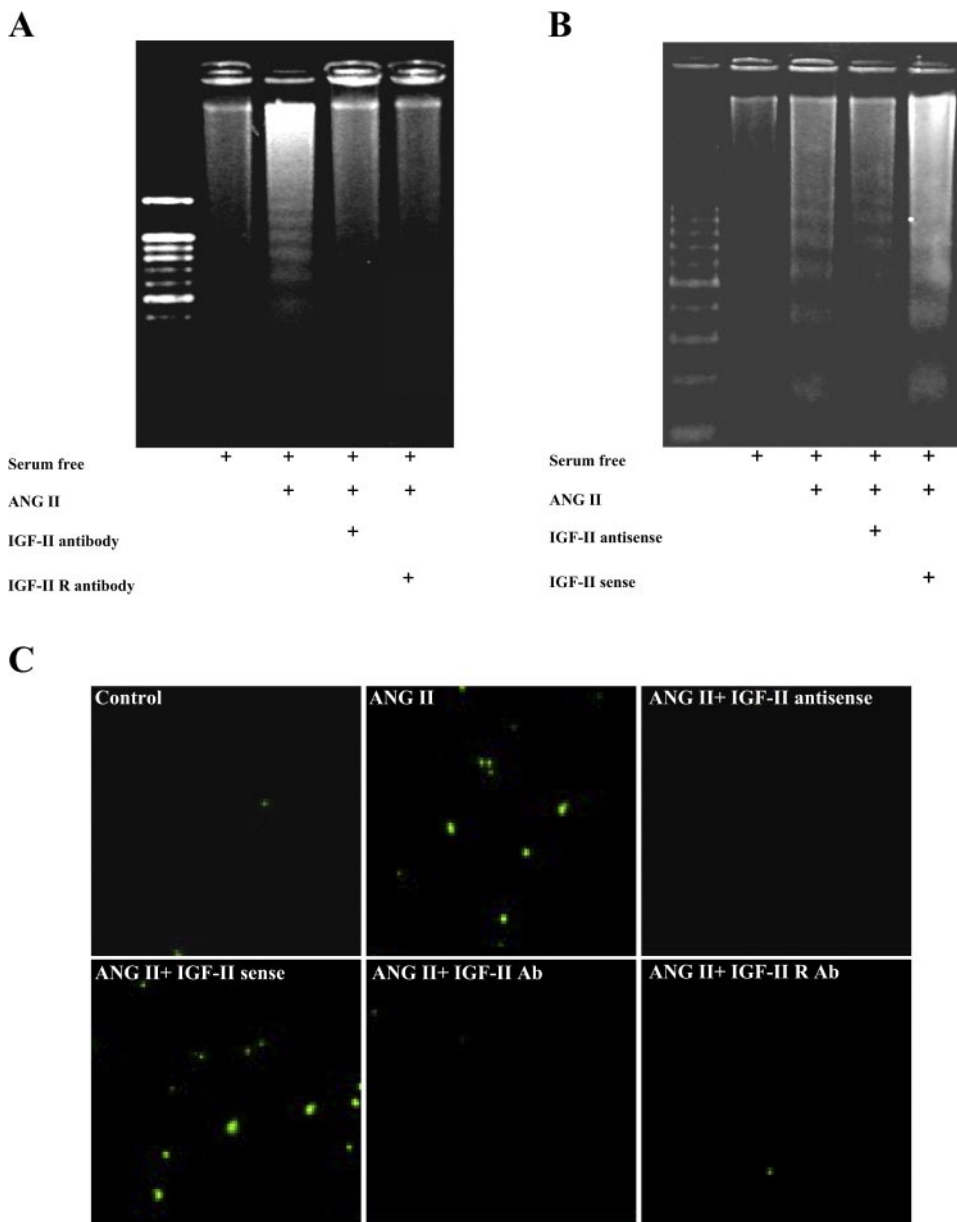


Fig. 1. ANG II-induced cardiomyoblast apoptosis in H9c2 cells was attenuated by IGF-II antibody, IGF II receptor (IGF-IIR) antibody, and antisense IGF-II. Representative DNA fragmentation and cellular apoptosis (repeated 3 times) was measured by 1.5% agarose gel electrophoresis, and TUNEL assay was used in cardiomyoblast H9c2 cells cultured in serum-free medium for 12 h and then treated with IGF-II antibody (IGF-II Ab, 100 ng/ml) or IGF-IIR antibody (IGF-IIR Ab, 100 ng/ml) and antisense IGF-II (14 μM) or sense IGF-II (14 μM) for 1 h and then treated with ANG II (10⁻⁸ M) or ANG II alone for 48 h.

CACCCTGTGCAA; rat GAPDH forward primer TCCCTCAAGAT-TGTCAGCAA, reverse primer AGATCCACAACGGATACA TT (MDBio, Taipei, Taiwan).

Protein Extraction and Western Blot Analysis

Cultured H9c2 cells were scraped and washed once with PBS. Cell suspension was then spun down, and cell pellets were lysed for 30 min in lysis buffer [50 mM Tris, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, pH 7.5, 10% glycerol, 1 mM basal medium Eagle, 1% Igepal-630, and proteinase inhibitor cocktail tablet (Roche)] and spun down 12,000 rpm for 10 min. Then, the supernatants were applied to new Eppendorf tubes for Western blot analysis. The protein extracts from the heart of rats with a complete ligation of the abdominal aorta were prepared by homogenizing the left ventricle samples in a PBS buffer (0.14 M NaCl, 3 mM KCl, 1.4 mM KH₂PO₄, 14 mM K₂HPO₄) at a concentration of 40 µg tissue/20 µl PBS for 5 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 rpm for 30 min. The supernatant was collected and stored at -70°C for further Western experiments. Proteins from the H9c2 cell line or animal heart extracts were then separated in 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific protein binding was

blocked in blocking buffer (5% milk, 20 mM Tris·HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) and blotted with specific antibodies of caspase 9, IGF-II, IGF-IIR, and α-tubulin (Santa Cruz Biotechnology) as indicated for each experiment in the blocking buffer at 4°C overnight. Densitometric analysis of immunoblots or PCR was performed using the AlphaImager 2200 digital imaging system (Digital Imaging System, San Leandro, CA).

Protocols

Protocol 1. To investigate whether ANG II-induced cardiomyoblast apoptosis is IGF-II system dependent, DNA fragmentation and apoptosis were measured by agarose gel electrophoresis and TUNEL assay in cardiomyoblast H9c2 cells treated with IGF-II antibody (100 ng/ml), IGF-IIR antibody (100 ng/ml), antisense IGF-II (14 µM), and sense IGF-II (14 µM) for 1 h and then treated with ANG II (10⁻⁸ M) for 48 h.

Protocol 2. To clarify whether and what pathways ANG II upregulates the IGF-II/IIR gene expressions, IGF-II/IIR RNAs were measured by RT-PCR in H9c2 cells pretreated with one of signaling pathway inhibitors individually, including SP-600125 (JNK inhibitor), CsA (calcineurin inhibitor), SB-203580 (p38 inhibitor), U-0126

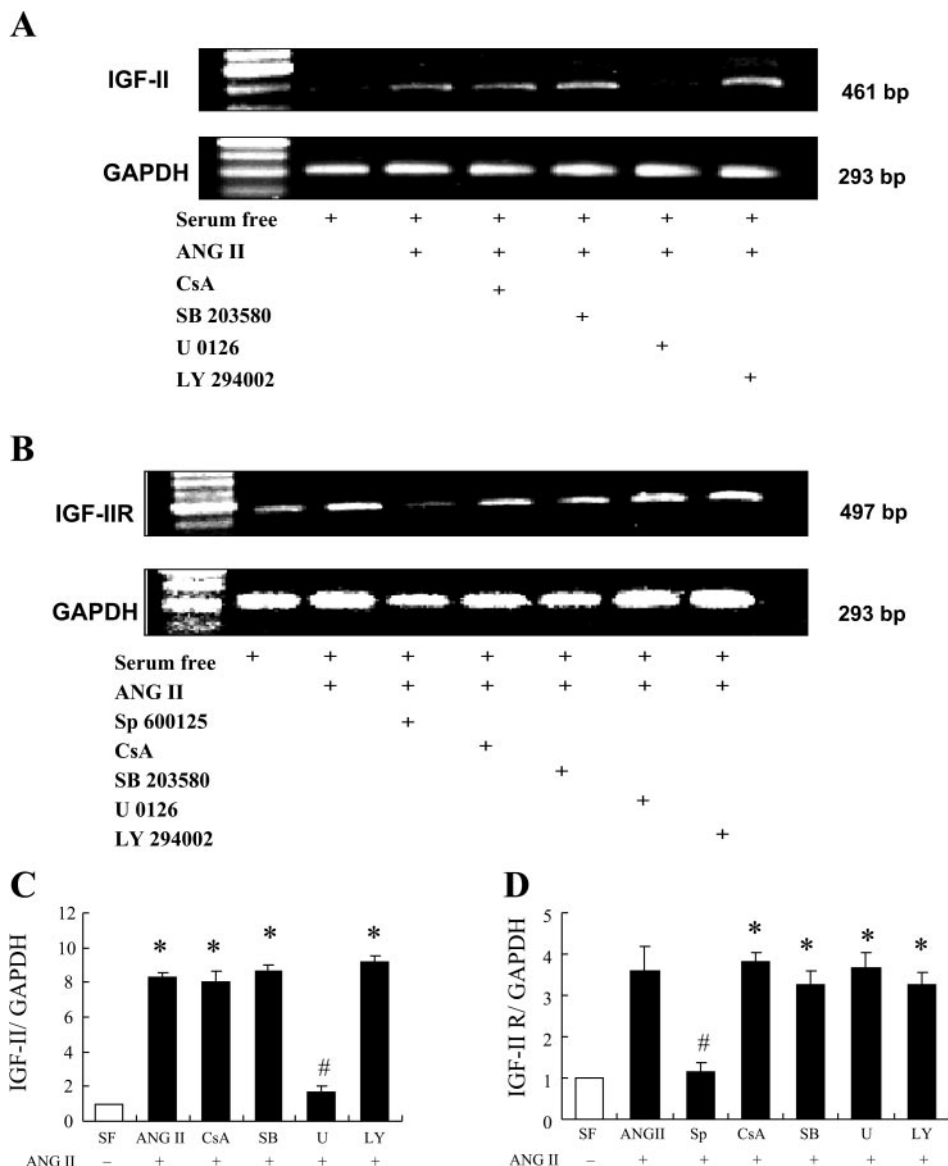


Fig. 2. Gene expressions of IGF-II and IGF-IIR induced by ANG II were mediated via ERK and JNK pathways, respectively, in H9c2 cells. *A*: representative amount of IGF-II and IGF-IIR gene expression (repeated 3 times) from extracted RNA were measured by RT-PCR in H9c2 cells cultured in serum-free (SF) medium for 12 h and then treated with one of the signaling pathway inhibitors individually, including CsA (calcineurin inhibitor), SP-600125 (SP, JNK inhibitor), SB-203580 (SB, p38 inhibitor), U-0126 (U, MEK inhibitor), or LY-294002 (LY, PI 3-kinase inhibitor). After 2 h, H9c2 cells were further treated with ANG II for 24 h. A relative quantification on the basis of GAPDH was applied. *B*: bars represent the relative quantification of IGF-II and IGF-IIR measured by densitometric analysis on the basis of *day 0* and indicate mean values ± SE (repeated 3 times). **P* < 0.05, significant differences from SF; #*P* < 0.05, significant differences from ANG II.

(MEK inhibitor), LY-294002 (PI 3-kinase inhibitor), and further treated with ANG II.

Protocol 3. To clarify what pathways ANG II induces apoptotic activity, DNA fragmentation was measured by agarose gel electrophoresis in H9c2 cells pretreated with one of the signaling pathway inhibitors, including SP-600125 (JNK inhibitor), CsA (calcineurin inhibitor), SB-203580 (p38 inhibitor), U-0126 (MEK inhibitor), and Staurosporine (PKC inhibitor) for 2 h and further treated with ANG II for 24 h.

Protocol 4. To clarify what pathways ANG II activates, proapoptotic protein caspase 8 and caspase 9 activity was measured by Western Blot analysis in H9c2 cells pretreated with one of the signaling pathway inhibitors individually, including SP-600125 (JNK inhibitor), CsA (calcineurin inhibitor), SB-203580 (p38 inhibitor), U-0126 (MEK inhibitor), and LY-294002 (PI 3-kinase inhibitor) for 2 h and further treated with ANG II for 24 h.

Protocol 5. To investigate whether the IGF II system and apoptosis-related proteins were upregulated in an animal model with elevated ANG II and hypertension, the mRNA expression and protein products of IGF-II/IIR and caspase 9 protein extracted from the left ventricle of excised hearts in 6-wk-old Sprague-Dawley rats with complete abdominal aorta ligation for 0, 1, 2, 3, 5, 7, 10, and 20 days were measured by RT-PCR and Western blot analysis.

Protocol 6. To investigate whether cardiac apoptosis occurs in an animal model with elevated ANG II and hypertension, cardiac tissues stained with TUNEL assay were measured in 6-wk-old Sprague-Dawley rats with complete abdominal aorta ligation for 0, 10, and 20 days.

Statistics

The blood pressures were compared between sham and ligation groups of animals from *day 0* to *day 20* using one-way analysis of variance (ANOVA) with preplanned contrast comparison against control level at the same day of postsurgery. Densitometric analysis of immunoblots or PCR in bar figures (Figs. 2, C and D, 4C, and 5C) were analyzed via ANOVA with preplanned contrast comparison against the control group (serum free) or against the ANG II group. The percentage of TUNEL-positive cardiac myocytes (Fig. 6B) were analyzed via ANOVA with preplanned contrast comparison against control level (*day 0*). In all cases, a difference at $P < 0.05$ was considered statistically significant. All data presented in the text and figures are means \pm SE.

RESULTS

ANG II-Induced DNA Fragmentation Is Attenuated by Blocking the IGF-II System

ANG II-induced DNA fragmentation was markedly attenuated by IGF-II antibody, IGF-IIR antibody (Fig. 1A), or antisense IGF-II, but not by sense IGF-II (Fig. 1B). ANG II-induced H9c2 cellular apoptosis measured by TUNEL assay was attenuated by IGF-II antibody, IGF-IIR antibody, or antisense IGF-II, but not by sense IGF-II (Fig. 1C). The findings in Fig. 1 suggest that ANG II-induced cardiomyoblast apoptosis was attenuated by blocking the IGF-II system.

Gene Expressions of IGF-II/IIR Induced by ANG II Were Mediated via ERK and JNK Pathways, Respectively, in H9c2 Cells

RT-PCR revealed an increase in the expression of IGF-II gene (Fig. 2A) and IGF-IIR gene (Fig. 2B) in H9c2 cells after administration of ANG II (10^{-8} M). The amount of gene expression in IGF-II and IGF-IIR was significantly increased by pretreatment of ANG II only (Fig. 2). ANG II-induced

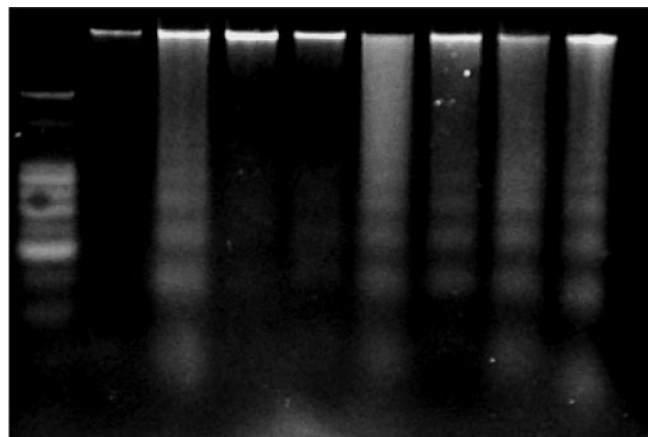
IGF-II gene overexpression was not attenuated by CsA, SB-203580, or LY-294002, but was attenuated by U-0126 (Fig. 2, A and C). ANG II-induced IGF-IIR gene overexpression was not attenuated by CsA, SB-203580, U-0126, or LY-294002, but was attenuated by SP-600125 (Fig. 2, B and D). The findings in Fig. 2 suggest that ANG II-induced IGF-II and IGF-IIR gene overexpression in cardiomyoblast was mainly via the ERK and JNK pathways, respectively.

ANG II-Induced Apoptosis in H9c2 Cells Was Activated by JNK, ERK, and Calcineurin Pathways

The increased DNA ladder formation revealed that cardiomyoblast H9c2 cells undergoing DNA fragmentation upon exposure to ANG II (10^{-8} M) was markedly attenuated by SP-600125 and CsA, and it was partially attenuated by U-0126 but was not attenuated by SB-203580, Staurosporine, or LY-294002 compared with the serum-free negative control (Fig. 3). The findings in Fig. 3 suggest that ANG II-induced cardiomyoblast apoptosis is mediated mainly by the JNK and calcineurin pathways and partially mediated by the ERK pathway.

ANG II-Induced Caspase 8 and Caspase 9 in H9c2 Cells Was Mediated by JNK, ERK, and Calcineurin Pathways

Compared with the serum-free negative control, the activity of the active form of caspase 8 (Fig. 4A) and caspase 9 (Fig. 4B) was increased, but the proform of caspase 8 (Fig. 4A) was decreased after administration of ANG II (10^{-8} M).



Serum free	+	+	+	+	+	+	+	+
ANG II		+	+	+	+	+	+	+
Sp 600125			+					
CsA				+				
SB 203580					+			
U 0126						+		
Staurosporine							+	+

Fig. 3. Possible pathways associated with ANG II-induced DNA fragmentation in H9c2 cardiomyoblasts. Representative DNA fragmentation (repeated 3 times) was measured by 1.5% agarose gel electrophoresis in cardiomyoblast H9c2 cells cultured in serum-free medium for 12 h and then treated with ANG II (10^{-8} M) alone or ANG II plus one of signaling pathway inhibitors individually, including SP-600125 (JNK inhibitor), CsA (calcineurin inhibitor), SB-203580 (p38 inhibitor), U-0126 (MEK inhibitor), or Staurosporine (PKC inhibitor, 10 μ M) for 24 h.

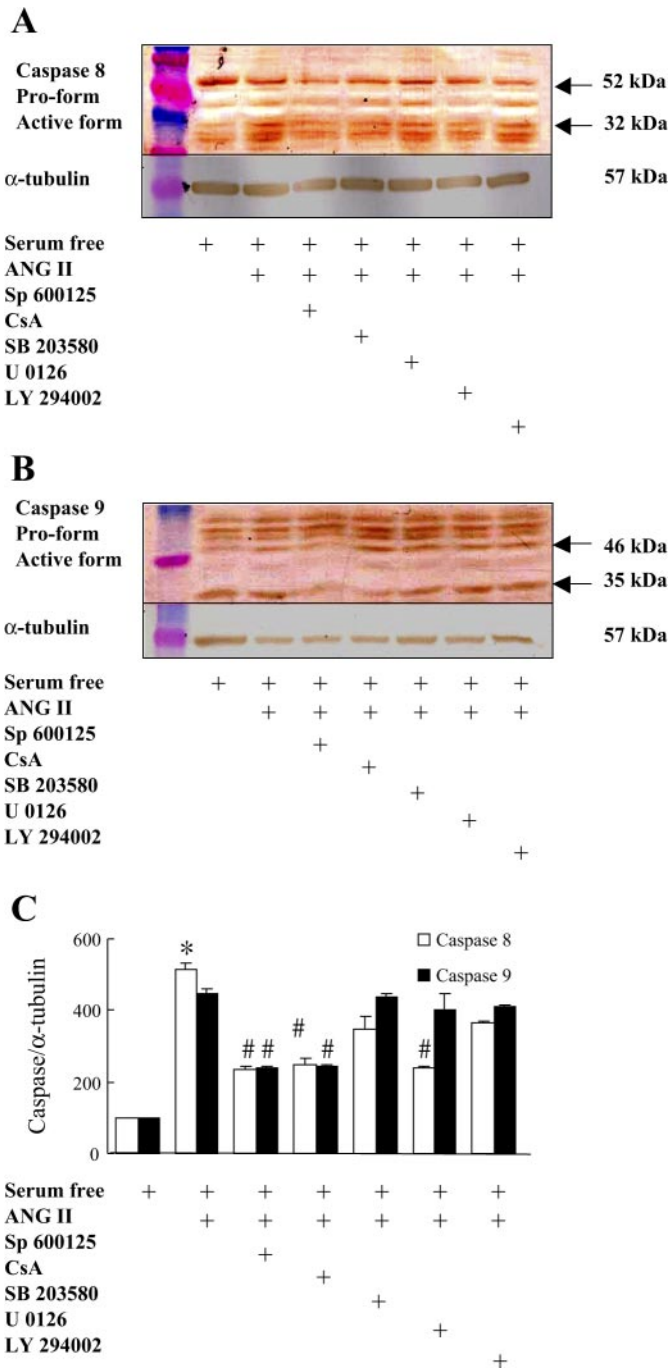


Fig. 4. Signaling pathways of caspase 8 and caspase 9 induced by ANG II in H9c2 cardiomyoblast cells. *A* and *B*: representative protein products of pro-form and active form of caspase 8 and caspase 9 (repeated 3 times) were measured by Western blotting analysis in H9c2 cells cultured in serum-free medium for 12 h and then treated with one of the signaling pathway inhibitors individually, including SP-600125, CsA (calcineurin inhibitor), SB-203580 (p38 inhibitor), U-0126 (MEK inhibitor), and LY-294002 (PI 3-kinase inhibitor). After 2 h, H9c2 cells were further treated with ANG II (10^{-8} M) for 24 h. Relative quantification on the basis of α -tubulin (40 μ g) was applied. *C*: bars represent relative quantification of active-form caspases 8 and 9 to α -tubulin on the basis of control group (serum free) and indicate mean values \pm SE (repeated 3 times). * P < 0.05, different from serum-free group; # P < 0.05, significant differences from ANG II group.

The increased activity of activated caspase 8 induced by ANG II was not affected by SB-203580 or LY-294002 but was attenuated by SP-600125, CsA, and U-0126 (Fig. 4, *A* and *C*). The increased caspase 9 activity induced by ANG II was not affected by SB-203580, U-0126, or LY-294002 but was attenuated by SP-600125 and CsA (Fig. 4, *B* and *C*). The findings in Fig. 4 suggest that ANG II-induced caspase 8 activity is mediated mainly by the JNK, calcineurin, and ERK pathways, whereas ANG II-induced caspase 9 activity

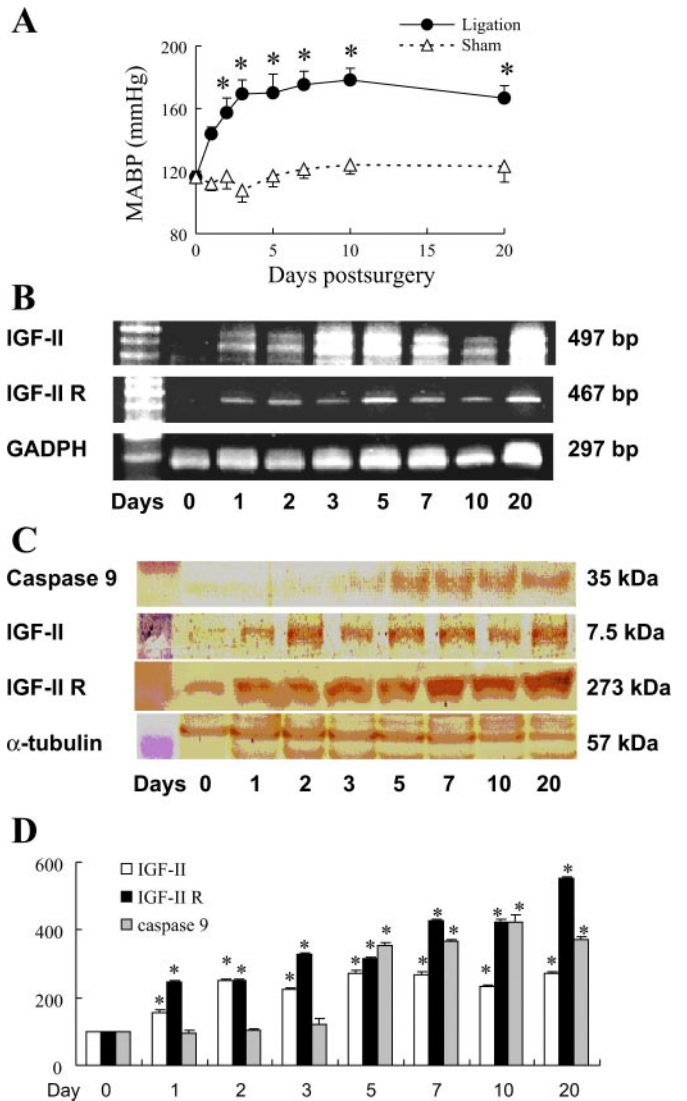


Fig. 5. Mean arterial blood pressure in rats with complete abdominal aorta ligation and IGF-IIR, IGF-II, and caspase 9 in hearts. *A*: mean arterial blood pressure (MABP) in 6-wk-old Sprague-Dawley rats with either sham or complete abdominal aorta ligation for 0, 1, 2, 3, 5, 7, 10, or 20 days were measured by tail cuff. * P < 0.05 significant difference between sham group and ligation group. *B*: gene expressions of IGF-II and IGF-IIR extracted from left ventricles of excised hearts in rats with complete abdominal aorta ligation from *days 0–20* were measured by RT-PCR. Relative quantification on the basis of GAPDH was applied. *C*: protein products of activated caspase 9, IGF-II, and IGF-IIR extracted from left ventricles of excised hearts in rats with complete abdominal aorta ligation from *days 0–20* were measured by Western blotting analysis. Relative quantification on the basis of α -tubulin was applied. *D*: bars represent relative quantification of IGF-II, IGF-IIR, and activated caspase 9 on the basis of *day 0* and indicate mean values \pm SE (repeated 3 times). * P < 0.05, significant differences from *day 0*.

is mainly mediated by the JNK and calcineurin pathways (Fig. 4C).

IGF-II, IGF-IIR, Caspase 9 Activity, and Mean Arterial Blood Pressure Were Increased in Rats with Complete Abdominal Aorta Ligation

Systolic blood pressure (not shown), diastolic blood pressure (not shown), and mean arterial blood pressure were all significantly increased following *days 2, 3, 5, 7, 10, and 20* and reached a plateau at *day 5* after complete abdominal aorta ligation compared with sham (Fig. 5A). The time-dependent increases in IGF-II/IIR gene expressions and protein products in the left ventricle were accompanied by an increase in activated caspase 9 protein levels and increased mean arterial blood pressure following *days 5, 7, 10, and 20* of complete abdominal aorta ligation. On the 20th day of ligation, IGF-II/IIR levels and caspase 9 activity both reached the highest levels. The percentage of TUNEL-positive cardiac myocytes in hearts excised from rats with complete abdominal aorta ligation was significantly increased on *day 10* and *day 20* compared with that on *day 0* (sham). The findings in Fig. 6 suggest that, in the rats with complete abdominal aorta ligation, the increased levels of IGF-II and IGF-IIR in the left ventricle

were accompanied by increases in caspase 9 activities and apoptosis after ligation.

DISCUSSION

Our main findings can be summarized as follows. 1) ANG II-induced DNA fragmentation and cellular apoptosis were markedly attenuated by IGF-II antibody, IGF-IIR antibody, and antisense IGF-II, but not by sense IGF-II, in H9c2 cells (Fig. 1). 2) The increased gene expressions of IGF-II and IGF-IIR induced by ANG II were markedly attenuated by U-0126 (MEK inhibitor) and SP-600125 (JNK inhibitor), respectively (Fig. 2). 3) DNA fragmentation and activated caspase 8 activity induced by ANG II were also attenuated by U-0126 (MEK inhibitor), SP-600125 (JNK inhibitor), and CsA (calcineurin inhibitor) in H9c2 cells, whereas activated caspase 9 activity induced by ANG II was only attenuated by SP-600125 (JNK inhibitor) and CsA (calcineurin inhibitor) (Figs. 3 and 4). 4) In the hypertensive rats with complete abdominal aorta ligation, the time-dependent increases in the gene expression and the protein levels of IGF-II and IGF-IIR in the left ventricles were accompanied by increases in activated caspase 9 activity, TUNEL-positive cardiac cell apoptosis, and blood pressure. In addition, the levels of IGF-II and IGF-IIR, the

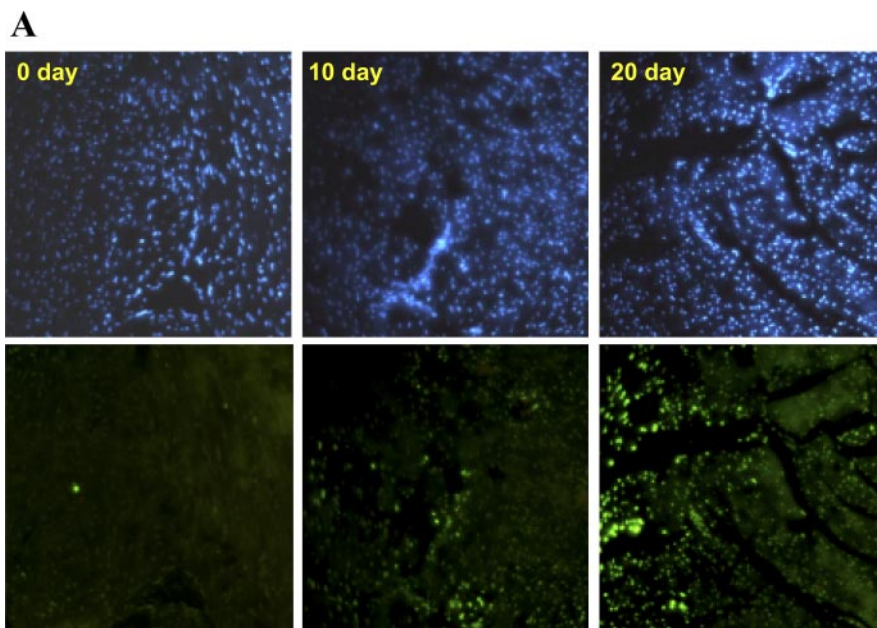


Fig. 6. A: total cells and apoptotic cells of excised hearts in 6-wk-old Sprague-Dawley rats with complete abdominal aorta ligation on *days 0, 10, and 20* were stained with 4,6-diamidino-2-phenylindole (DAPI) (*top*, blue spots) and TUNEL assay (*bottom*, green spots), respectively. B: bars represent the percentage of TUNEL-positive cardiac myocytes on the basis of total cells stained with DAPI and indicate mean values \pm SE (repeated 3 times). * $P < 0.05$, significant differences from *day 0*.

level of activated caspase 9, and the number of cardiac TUNEL-positive cells reached much higher levels on the 20th day after ligation (Fig. 5). To our knowledge, this study is the first time to identify the roles of IGF II and IIR in ANG II-induced cardiomyoblast apoptosis and in rat heart with abdominal aorta ligation. After integrating the current findings, we summarized the roles of IGF-II in ANG II-induced cardiomyoblast apoptosis and in rat heart with abdominal aorta ligation in Fig. 7.

ANG II markedly increased DNA fragmentation, caspase 8 activity, caspase 9 activity, and the number of TUNEL-positive cells in cardiomyoblast H9c2 cells. In addition, the increased caspase 9 activity and TUNEL-positive apoptosis were also found in the rat hearts with elevation of ANG II and blood pressure. These findings suggest that ANG II induces cardiomyoblast and cardiac cell apoptosis. ANG II appears to be able to induce apoptosis in diverse cellular types and to be a deliberate form of cell death involved in the pathogenesis of various cardiovascular diseases, including heart failure and vascular remodeling (4). ANG II has been reported to be involved in the stimulation of cardiac apoptosis in essential hypertension (9, 16). Apoptosis has been found to be preceded by increases in tissue ANG II but not in plasma ANG II concentrations in canine models of congestive heart failure (6). Therefore, the effects of ANG II on cardiac cells may be critical in the development of cardiovascular diseases and hypertension. The current study not only confirms that ANG II induces cardiomyoblast apoptosis but also reveals the IGF-II-related pathways for ANG II-induced cardiac apoptosis, a matter of great scientific interest.

In the current study, because ANG II-induced apoptotic DNA fragmentation and apoptosis in cardiomyoblast H9c2 cells appear to be attenuated by antisense IGF-II, IGF-II antibody, and IGF-IIR antibody, we can speculate whether IGF-II with its downstream

pathway activation may play a fundamental role in ANG II-induced cardiomyoblast apoptosis and cause DNA fragmentation. Surprisingly, when IGF-II and IGF-IIR were blocked, the deleterious action of ANG II appeared to be greatly suppressed in cardiomyoblast apoptosis. To our knowledge, this study is the first to report the inhibitory roles of IGF-II blockade and IGF-IIR antibody in ANG II-induced apoptosis in cardiac cells.

Because IGF-II and IGF-IIR play a critical role in ANG II-induced apoptosis in cardiomyoblast cells, it was critical to know whether ANG II would modulate the IGF-II system via certain signaling pathways. Our second major finding was that ANG II did upregulate the gene expressions of IGF-II and IGF-IIR and that the ANG II-induced the gene expressions of IGF-II and IGF-IIR were markedly attenuated by U-0126 (MEK inhibitor) and SP-600125 (JNK inhibitor), respectively. We can speculate that ANG II upregulates IGF-II gene expression by activating the ERK pathway, whereas ANG II upregulates IGF-IIR gene expression by activating the JNK pathway. To our knowledge, this is the first report showing that IGF-II and IGF-IIR are upregulated by ANG II via the ERK and JNK pathways, respectively, in cardiomyoblast cells.

DNA fragmentation, caspase 8 activity, caspase 9 activity, and cellular apoptosis induced by ANG II were attenuated by CsA (calcineurin inhibitor) in H9c2 cells, suggesting that ANG II-induced cardiomyoblast apoptosis was mediated not only by the IGF-II system described above but also by calcineurin-dependent pathways. As was found in our study, calcineurin inhibitor has been shown to inhibit ANG II-induced apoptosis in endothelial cells, suggesting that calcineurin inhibitor has a general apoptosis-suppressive effect (25). Because the unregulated gene expressions of IGF-II and IGF-IIR induced by ANG II were markedly attenuated by U-0126 (MEK inhibitor) and SP-600125 (JNK inhibitor), respectively, we wondered whether ANG II-induced apoptosis would be mediated by upregulation of the IGF-II system via ERK and JNK signaling pathways. In the current study, DNA fragmentation and caspase 8 activity induced by ANG II were also attenuated by SP-600125 (JNK inhibitor), by CsA (calcineurin inhibitor), and partially by U-0126 (MEK inhibitor) in H9c2 cells, whereas caspase 9 activities induced by ANG II were attenuated only by SP-600125 (JNK inhibitor) and CsA (calcineurin inhibitor) (Figs. 3 and 4). In other words, after blocking IGF-IIR and the JNK pathway, activated caspase 8 and caspase 9 apoptotic protein levels were both attenuated, but after blocking IGF-II and the MEK pathway, only activated caspase 8 (not activated caspase 9) was attenuated. In addition, when calcineurin was blocked, the effects of ANG II were largely inhibited. IGF-IIR is a multifunctional single-transmembrane glycoprotein that has been shown to bind both IGF-II and mannose 6-phosphate (8). If the action of IGF-II is blocked, another IGF-IIR ligand, mannose 6-phosphate, or other ligands may also have some unknown effects on IGF-IIR, possibly explaining why blockade of IGF-II decreases only caspase 8 (not caspase 9), unlike the blockade of IGF-IIR. However, very little is known about the physiological significance of mannose 6-phosphate on IGF-IIR in the function of the cardiovascular system. In sum, ANG II appears to upregulate IGF-II and IGF-IIR via the ERK and JNK signaling pathways, respectively, and further activates caspase 8 and caspase 9 via calcineurin-dependent pathways.

In the current study, the time-dependent increases in the gene expressions and protein products of IGF-II and IGF-IIR in the left ventricle were accompanied by an increase in activated caspase 9

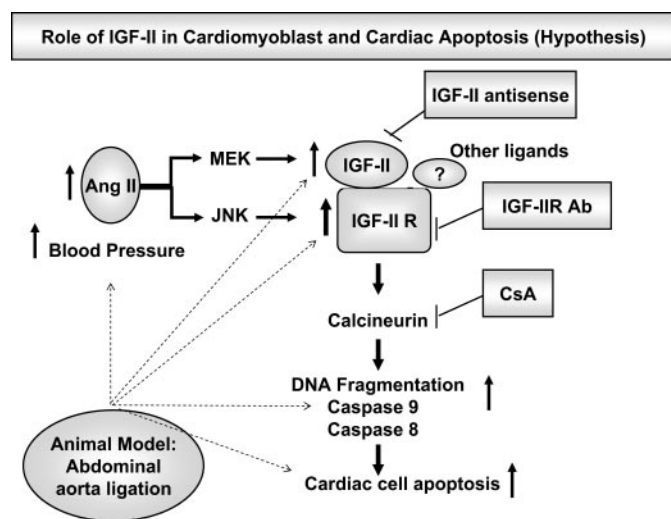


Fig. 7. Possible roles and pathways of IGF-II and IGF-IIR in ANG II-induced cardiomyoblast and in hypertensive rats with abdominal aorta ligation. ANG II-induced cardiomyoblast apoptosis was reversed by blockades of IGF-IIR signaling such as IGF-II blockade and IGF-IIR blockade. ANG II-induced cardiomyoblast apoptosis coexisted with increased transactivation of IGF-II and IGF-IIR, which are mediated by ERK and JNK pathways, respectively, both of which further contribute to cardiomyoblast apoptosis via calcineurin signaling. Additionally, the findings in the ANG II-elevated and hypertensive animal models with complete abdominal aorta ligation partially support our hypothesis and show that ANG II upregulated IGF-II and IGF-IIR expression and induced activated caspase 9-related apoptotic activities in rat hearts.

protein levels and TUNEL-positive cardiomyocytes following 0–20th day of complete abdominal aorta ligation, suggesting that the IGF II system and apoptotic activity are upregulated in animal models with elevated ANG II and hypertension. The findings in the animal models that we used support our current findings that ANG II induced caspase 9 activities and cellular apoptosis and that ANG II upregulated the gene expression of IGF-II and IGF-IIR in cardiac cells and rat hearts. The increase of plasma ANG II and the changes of myocardial tissues have been previously reported in the rat model with abdominal aortic ligation around the aorta above and below the renal arteries (5, 10). In another previous study, abdominal aortic ligation increased the ratio of heart weight-to-body weight ~22% and increased apoptotic cardiocytes after 2 wk, suggesting that pressure overload coexisting with elevated ANG II may contribute to chronic hypertrophy and cardiocyte apoptosis in rat hearts (22). One study has mentioned that the transcript levels for IGF-II and their associated cell surface receptors were increased in banded suprarenal abdominal aorta of 5-day-old rat pups during the early neonatal periods of heart development (11). This indirectly supports our findings that the upregulation of the gene expressions and protein products of IGF-II and IGF-IIR in the hearts excised from rats with complete abdominal aorta ligation.

Significance in Clinical Application

Prior to our studies, very limited information was available about how IGF-II and IGF-IIR contribute to ANG II-induced cardiac apoptosis. ANG II has been found to play a critical role in controlling cardiovascular and renal homeostasis, whereas unbalanced (overactivated) ANG II is known to contribute to cardiovascular diseases such as hypertension, atherosclerosis, and heart failure (13). Our findings may provide direct evidence that myocardial cell death in cardiovascular patients in coexistence with an elevation of ANG II level might be caused by an upregulation of the IGF-II system via the ERK and JNK signaling pathways and calcineurin activity.

With regard to therapeutic application, our findings may further propose that cardiovascular patients should normalize ANG II function in cardiac tissues, such as by applying ANG II inhibitor and angiotensin-converting enzyme (ACE) inhibitor as well as proposing that cardiovascular patients should prevent an upregulation of the IGF-II system and calcineurin activity, such as by applying IGF-II, IGF-IIR, or calcineurin inhibitors to suppress cardiac apoptosis. Of course, further studies are required to clarify the possible clinical intervention related to the mechanism of the IGF-II system in the ANG II-induced cardiac apoptosis.

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