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# Cardiomyoblast apoptosis induced by insulin-like growth factor (IGF)-I resistance is IGF-II dependent and synergistically enhanced by angiotensin II

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**Abstract** *Objective:* This study explores the synergistic effect of cardiomyoblast apoptosis induced by angiotensin II (Ang II) and Insulin-like growth factor (IGF)-I resistance, and elucidates the role of IGF-II via IGF-II receptor (R) and calcineurin pathways in apoptosis induced by Ang II and IGF-I resistance. *Methods*: Apoptosis of cultured cardiomyoblast H9c2 cells was assessed by DNA fragmentation on agarose gel electrophoresis, nuclear condensation stained with DAPI, and Western blot analysis of pro-apoptotic Bad and cytochrome *c* in various combinations of control, Ang II, antisense IGF (I or II), IGF (I or II) antibody, IGF (I or II) receptor (R) antibody, or calcineurin inhibitor (Cyclosporine A, (CsA)). *Results*: We found the following: (1) The combination of Ang II and IGF-I deficiencies had a synergistic effect on apoptosis, confirmed by DNA fragmentation, nuclei condensation, and increases in such pro-apoptotic proteins as Bad, cytochrome *c*, caspase 9, and caspase 3 in H9c2 cells. (2) IGF-II and IGF-IIR protein products were increased by antisense IGF-I and IGF-I resistance, but these IGF-II protein products were not affected by sense IGF-I and non-specific antibody IgG in H9c2 cells. (3) The alteration of Bad protein level and the release of cytochrome *c*, both induced by treatments containing combinations of Ang II and antisense IGF-I, IGF-I antibody or IGF-IR antibody, were inhibited by IGF-II antibody. (4) DNA fragmentation, Bad, and cytochrome *c* which was induced by treatments combining IGF-IR antibody with Ang II or combining IGF-IR antibody with IGF-II were remarkably attenuated by CsA. *Conclusion*: IGF-I deficiency and/or IGF-IR resistance induced apoptosis in cardiomyoblast cells. The apoptosis, which might have been caused by the upregulation of IGF-II and IGF-IIR genes possibly activated the downstream calcineurin pathway, was synergistically augmented by Ang II.

**Keywords** IGF-I resistance · Angiotensin II · Calcineurin · IGF-II · Cardiomyoblast apoptosis

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## Introduction

Apoptosis, which is characterized by DNA-fragmentation, nuclear condensation, alterations in pro-apoptotic proteins levels, such as Bad, and release of mitochondrial cytochrome *c* into the cytoplasm, may contribute to many cardiac

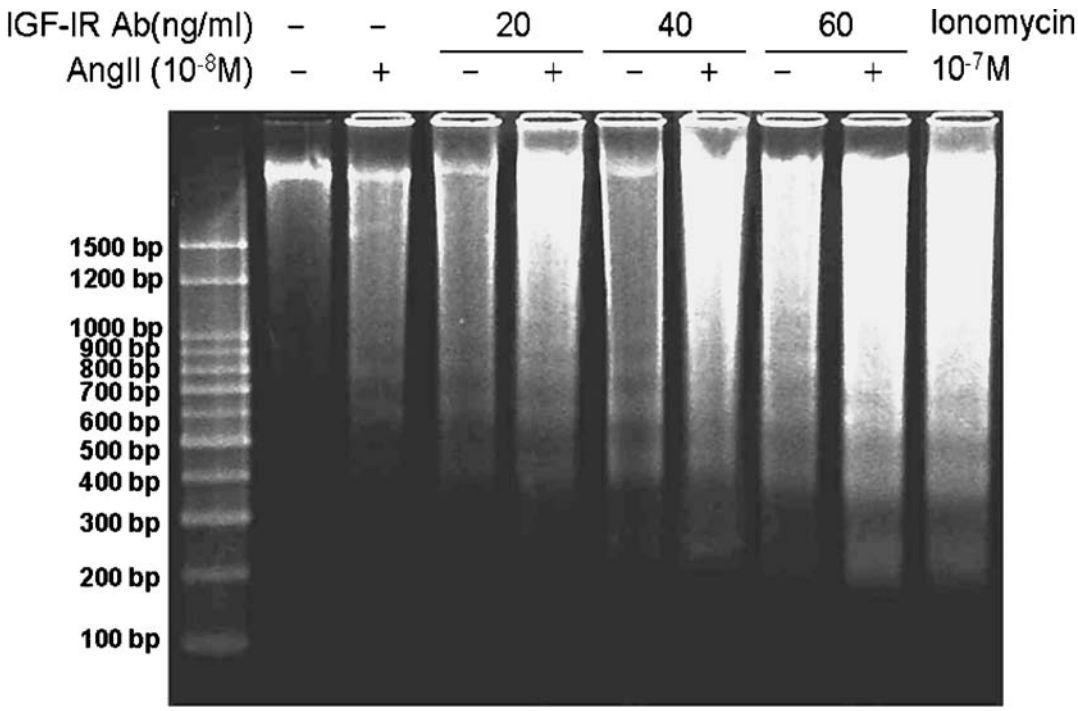


**Fig. 1** DNA fragmentation was induced by Ang II and/or IGF-I receptor antibody. H9c2 cells were cultured in serum-free medium as negative control for overnight then treated with Ang II ($10^{-8}$ M) and/or IGF-I receptor antibody (20, 40, 60 ng/ml). The incubation was continued for another 24 h, and then the cells were harvested, extracted, and DNA fragmentation was determined. Cells were treated with ionomycin ($10^{-7}$ M) as positive control ($n = 3$)

disorders [1, 2]. An increase in levels of the pro-apoptotic protein angiotensin II (Ang II) has been observed in hypertension, coronary artery disease, left ventricular hypertrophy, and heart failure [3] and may function as a regulator of apoptosis in cardiac tissues [1]. One pathway of angiotensin II induced hypertrophy and apoptosis may be calcium-calcineurin [4]. Besides, patients with diabetes mellitus often exhibit abnormalities in insulin-like growth factor (IGF)-I, such as reduced circulating levels of IGF-I [5] and increased IGF-I resistance [6]. IGF-I has been found to improve myocardial function, promote cardiac growth, improve cardiac contractility, evoke ventricular remodeling, and prevent heart failure [7]. IGF-I has also been found to suppress myocardial apoptosis and exert an anti-apoptotic effect in cardiac cells through the phosphatidylinositol 3-kinase/Akt kinase pathway [8, 9]. The cardiac survival or apoptotic levels appear to be directly related to the amount of IGF-I expressed [10, 11]. However, while IGF-I and Ang II seem to play a role in cardiac apoptosis, it is unknown whether their combined effect is non-additive, sub-additive, additive, or synergistic.

IGF-I receptor (R) can be activated by IGF-I, IGF-II, and insulin at supraphysiological concentrations. The IGF II is one of the ligands that share a common IGF-IR signaling pathway [11, 12]. The IGF-IR signaling pathway appears to play an important role in the suppression of apoptosis in a variety of cells, including cardiac cells [10, 11]. However, when the IGF-I pathway is blocked, IGF-II may stimulate myocardiac cell hypertrophy through the IGF-IIR signaling pathway [13], which includes Gi-protein and calcium-calcineurin pathways [14, 15]. Although the exact mechanism of myocardiac hypertrophy and apoptosis is not totally correlated, Angiotensin-II activate calcium-calcineurin pathway is both involved in hypertrophy [16] and apoptosis [17]. However, the apoptotic mechanism of IGF-I and IGF-II is unclear. With regard to cardiac cell death, it is unknown whether IGF-I resistance will increase IGF-IIR and IGF-II protein products and further activate IGF-IIR signaling via calcineurin pathway.

To clarify the answers to these questions, this study assesses apoptosis of cultured cardiomyoblast H9c2 cells. IGF-I deficiency and/or IGF-IR resistance was found to induce cardiomyoblast apoptosis, which might have been caused by an upregulation of IGF-II and IGF-IIR genes with its downstream calcineurin pathway activation. This apoptosis was possibly synergistically augmented by Ang II.

## Methods

### Cell culture

H9c2 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 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 1 mM pyruvate in humidified air ($CO_2$ 5%) at 37°C. H9c2 cells were cultured in serum-free medium with minimal essential medium. for 12 h, and then treated with or without Ang II ($10^{-8}$ M) (Sigma Chemical Co., St. Louis, MO, USA), antisense IGF-I (14 $\mu$M), antisense IGF-II (14 $\mu$M), IGF-I receptor antibody (100 ng/ml) (Neo Markers, Fremont, CA, USA) or IGF-II antibody (100 ng/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The incubation was continued for another 24 h, and then the cells were harvested and extracted for the analysis.

## Design and synthesis of IGF-I and IGF-II oligonucleotides

The 15-mers of IGF-I and IGF-II antisense oligonucleotides (AsONs) used in this study are known to target the translation initiation site of IGF-I and IGF-II mRNAs in rats, and are complementary to nucleotides (5′-ATG TCG TCT TCA CAT-3′ and 5′-ATG GGG ATC CCA GTG-3′). In addition, the IGF-I sense oligonucleotides, which are the same as nucleotides (5′-ATG TCG TCT TCA CAT-3′ and 5′-ATG GGG ATC CCA GTG-3′ were used as negative controls The sequences had no similarity to other mammalian genes. All antisense 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)) and stored at −20°C.

## Transfection of H9c2 cells using LipofectAMINE

Cells were grown to 80% confluence in 100-mm dishes. IGF-I or IGF-II oligonucleotides and l ipofectAMINE were separately diluted in serum-free DMEM to 200-$\mu$l 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.

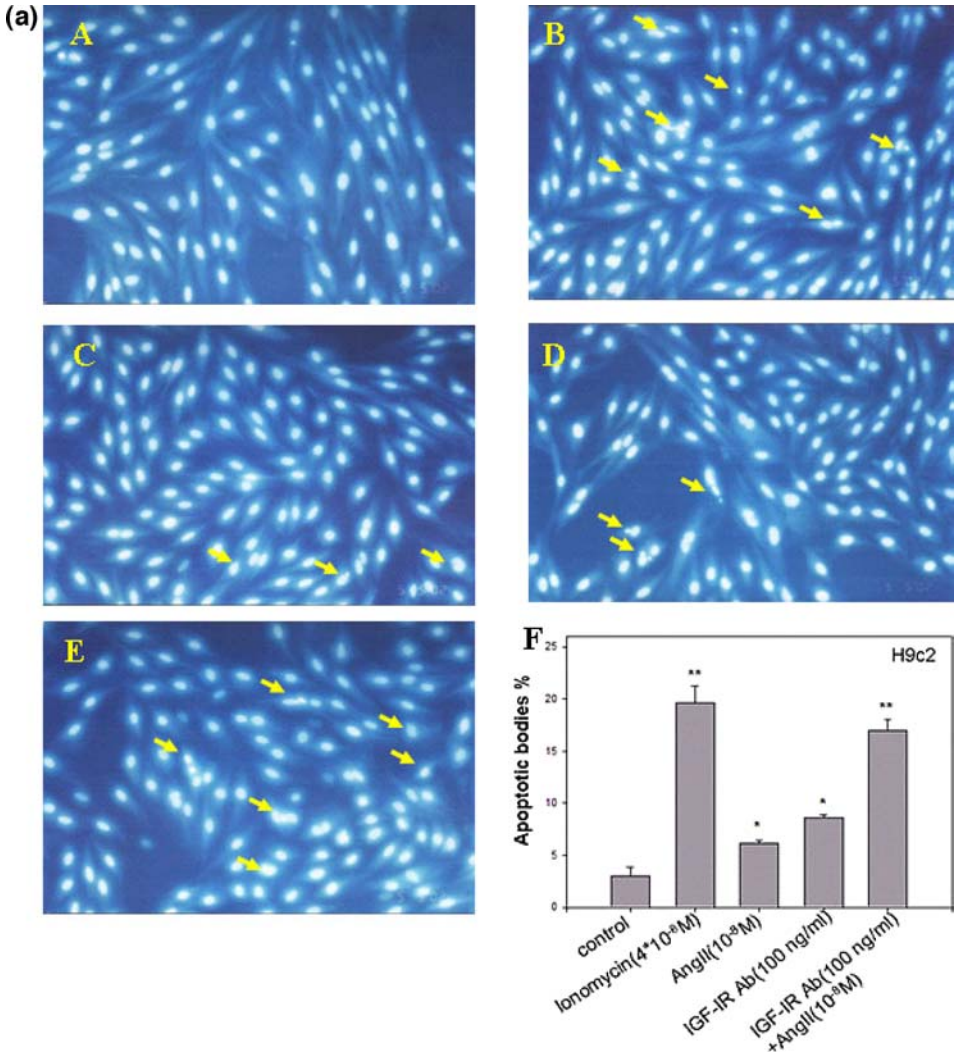


**Fig. 2** Cardiomyoblast apoptosis was synergistically induced by Ang II and IGF-IR antibody. H9c2 cells were cultured in serum-free medium for overnight and then treated with Ang II ($10^{-8}$ M) and/or IGF-I receptor antibody (100 ng/ml). The incubation was continued for another 24 h. DAPI (4,6-diamidino-2-phenylindole) (1 $\mu$g/ml, Fig. (a)) staining and cleaved caspase 3 and TUNEL assay (Fig. (b)) were determined. Cells were treated with ionomycin ($4 \times 10^{-8}$M) as positive control. A, serum-free medium; B, ionomycin; C, Ang II; D, IGF-IR antibody; E, Ang II plus IGF-IR antibody; F, the quantitive results. The values are the mean ± S.E. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$ versus the cells treated with serum-free medium. Arrow head indicated the nuclei condensed cells. A relative quantification of caspase 3 on the basis of $\alpha$-tubulin was applied. Bars in Fig. (b) represent the percentage of TUNEL-positive cardiac myocytes on the basis of stained total cells by DAPI and indicate mean values ± SE ($n = 3$)

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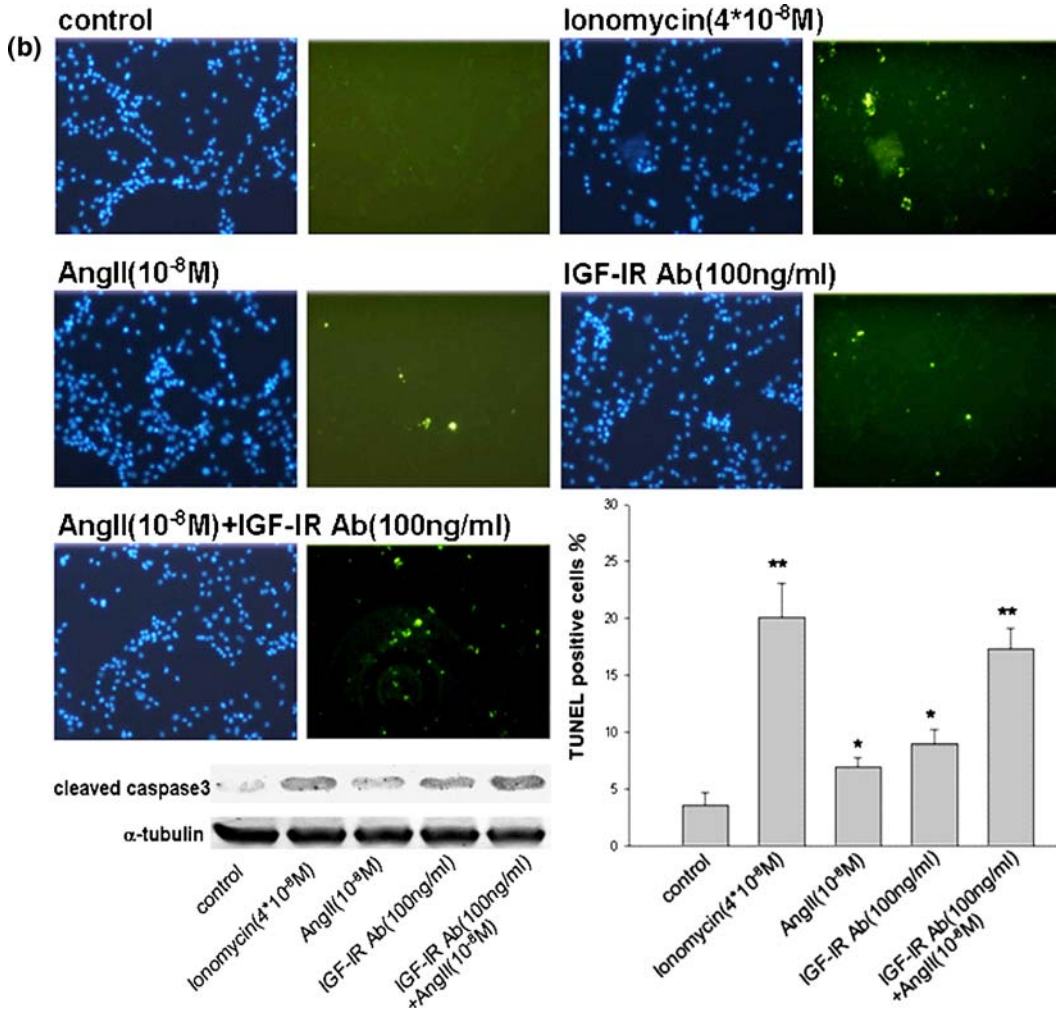


**Fig. 2** (Continued)

The cells were incubated at 37°C for 6 h, at which time the medium was replaced with DMEM-10% FBS. The cells were harvested after 24 h.

Agarose gel electrophoresis for DNA fragmentation

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

DAPI staining and TUNEL assay

After various treatments, H9c2 cells grown on 6 mm plate were fixed with 4% paraformaldehyde solution for 30 min at room temperature. After a rinse with phosphate-buffered saline, the samples were first incubated with phalloidin-rhodamine for 1 h and with Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL) reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP (Roche Applied Science, Indianapolis, IN, USA). The cells were also stained with 1 ug/ml 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI, Roche) for 30 min 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. Use 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 and number of apoptotic bodies were determined by counting $3 \times 10^5$ cardiac myocytes. All morphometric measurements were performed by at least two independent individuals in a blinded manner.

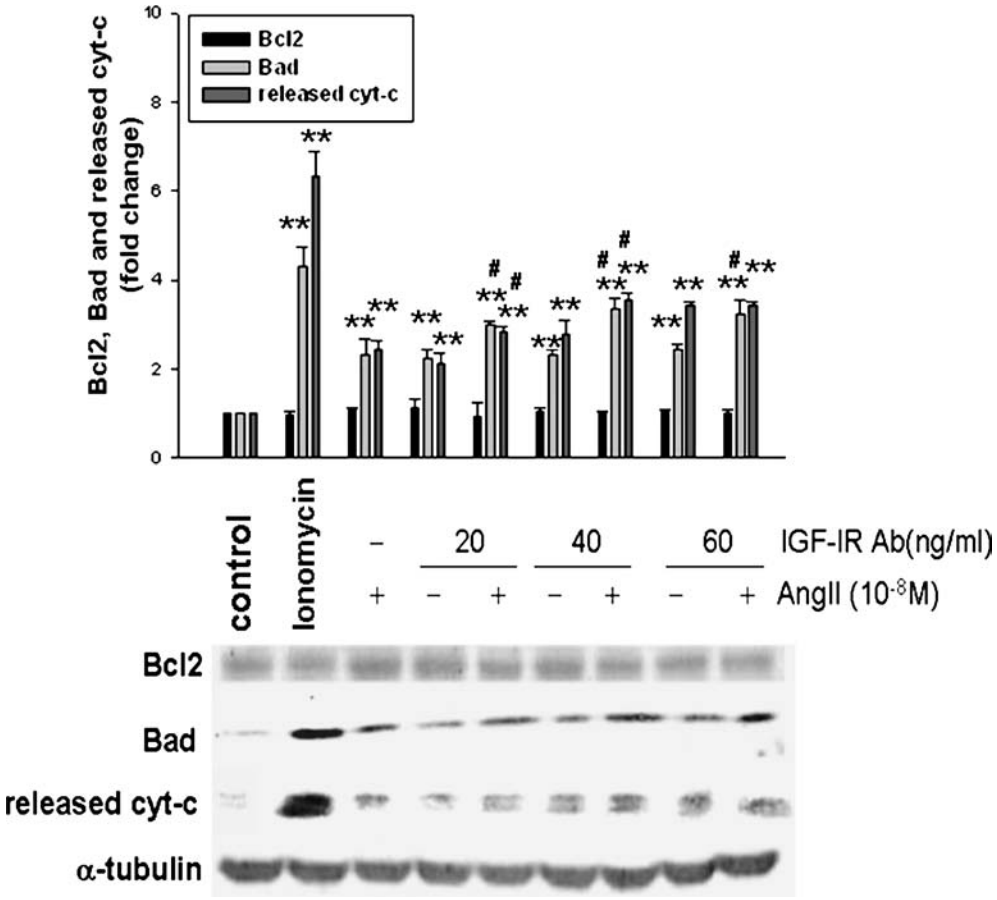


**Fig. 3** The amount of pro-apototic Bad protein and the release of cytochrome c were increased by Ang II and IGF-IR antibody. H9c2 cells were cultured in serum-free medium for overnight and then treated with Ang II ($10^{-8}$ M) and/or IGF-I receptor antibody (20, 40, 60 ng/ml). The incubation was continued for another 24 h, and the cells were harvested and extracted for the Western Blot analysis. Cells were treated with ionomycin ($10^{-7}$ M) as positive control. Bars represent the relative quantification of Bcl2, Bad, and released cytochrome *c* on the basis of control level and indicate mean fold change ± SE ($n = 3$). $^{**}$ $P < 0.01$, significant differences between groups with vs. without Ang II administration, $^{\#}P < 0.01$, significant differences between IGF-IR groups with vs. without Ang II administration

## Western blot analysis

Cultured H9c2 cells were scraped and washed once with phosphate-buffered saline. 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 BME, 1% IGEPAL-630 and proteinase inhibitor cocktail (Roche)) and spun down 12,000 rpm for 10 min in an Eppendorf tube. Proteins were then separated in 12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific protein binding was stopped 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 3, caspase 9 (Santa Cruz Biotechnology), Bcl2, Bad (BD Biosciences, Heidelberg, Germany), p-Bad, and cytochrome *c* (Cell Signaling Technology, Beverly, MA, USA) in the blocking buffer at 4°C overnight. For repeated blotting, nitrocellulose membranes were stripped with Restore Western blot stripping buffer (Pierce Biotechnology, Inc, Rockford, IL, USA) at room temperature for 30 min.

To detect cytochrome *c*, cells were suspended in a buffer (50 mM Tris (pH 7.5), 0.5 M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol and proteinase inhibitor cocktail table (Roche) for 3 min on ice, homogenized by 40 strokes in a Dounce homogenizer, and centrifuged at 12,000 rpm for 15 min. The supernatant was the cytosol fraction; the pellet was resuspended in lysis buffer as the membrane fraction.

## Statistical analysis

Statistical differences were assessed by one way-ANOVA. $P < 0.05$ was considered statistically significant. Data were expressed as the mean ± SEM.

## Results

### Apoptosis measured by DNA fragmentation was induced by Ang II and/or IGF-IR antibody

To clarify whether cardiomyoblast apoptosis was induced by Ang II and/or IGF-IR antibody, DNA fragmentations were measured with or without administration of Ang II ($10^{-8}$ M) and/or IGF-I receptor antibody (20, 40, 60 ng/ml). DNA ladder formation revealed that cardiac H9c2 cell underwent DNA fragmentation when exposed to either Ang II ($10^{-8}$ M) or IGF-IR antibody (20, 40, and 60 ng/ml), compared to the negative and positive controls (Fig. 1). We observed a synergistic effect rather than additive effect on DNA fragmentation with the combination of Ang II ($10^{-8}$ M) and IGF-IR antibody (20, 40, and 60 ng/ml)

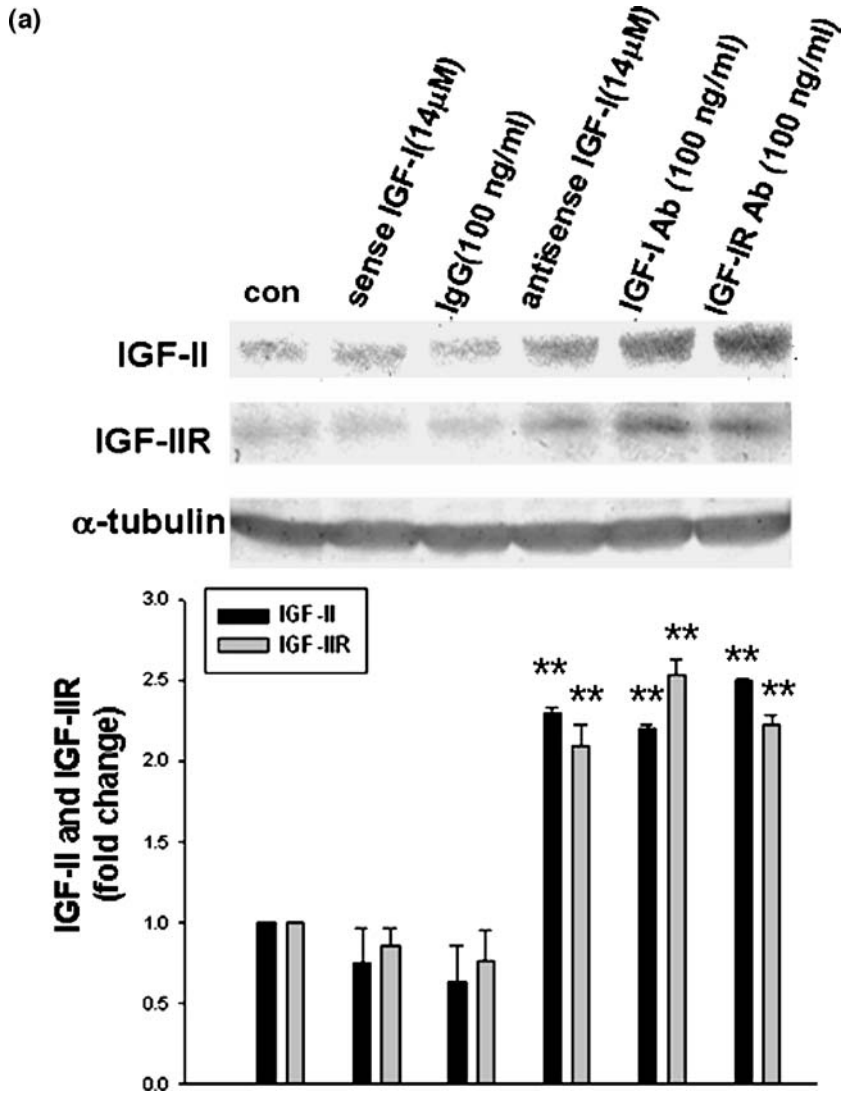


**Fig. 4** (a) The IGF-II and IGF-IIR protein products were induced by antisense IGF-I, IGF-IR antibody or IGF-I antibody. H9c2 cells cultured in serum-free medium for overnight then treated with antisense IGF-I (14 $\mu$M) and sense IGF-I (14 $\mu$M) as a negative control, as well as IGF-I receptor antibody (100 ng/ml), IGF-I antibody (100 ng/ml), and non-specific IgG (100 ng/ml) as a control antibody. The incubation was continued for another 24 h, and then the cells were harvested and extracted for the Western Blot analysis. Bars represent the relative quantification of IGF-II and IGF-II receptor on the basis of control level and indicate mean fold change $\pm$ SE ($n = 4$). ** $P < 0.01$, significant differences from control group. (b) Antisense IGF-I inhibit the IGF-I protein products. H9c2 cells cultured in serum-free medium for overnight then treated with sense IGF-I (14 $\mu$M), antisense IGF-I (7 $\mu$M) and antisense IGF-I (14 $\mu$M). Bars represent the relative quantification of IGF-I on the basis of control level and indicate mean fold change $\pm$ SE ($n = 4$). ** $P < 0.01$, significant differences from control group. (c) Antisense IGF-I and IGF-I antibody inhibit the phosphorylated Akt and Akt protein products. H9c2 cells cultured in serum-free medium for overnight then treated with antisense IGF-I (7 $\mu$M and 14 $\mu$M) and IGF-I antibody (20, 50, 100 ng/ml). Bars represent the relative quantification of phosphorylated Akt and Akt protein products on the basis of control level and indicate mean fold change $\pm$ SE ($n = 4$). ** $P < 0.01$, significant differences from control group

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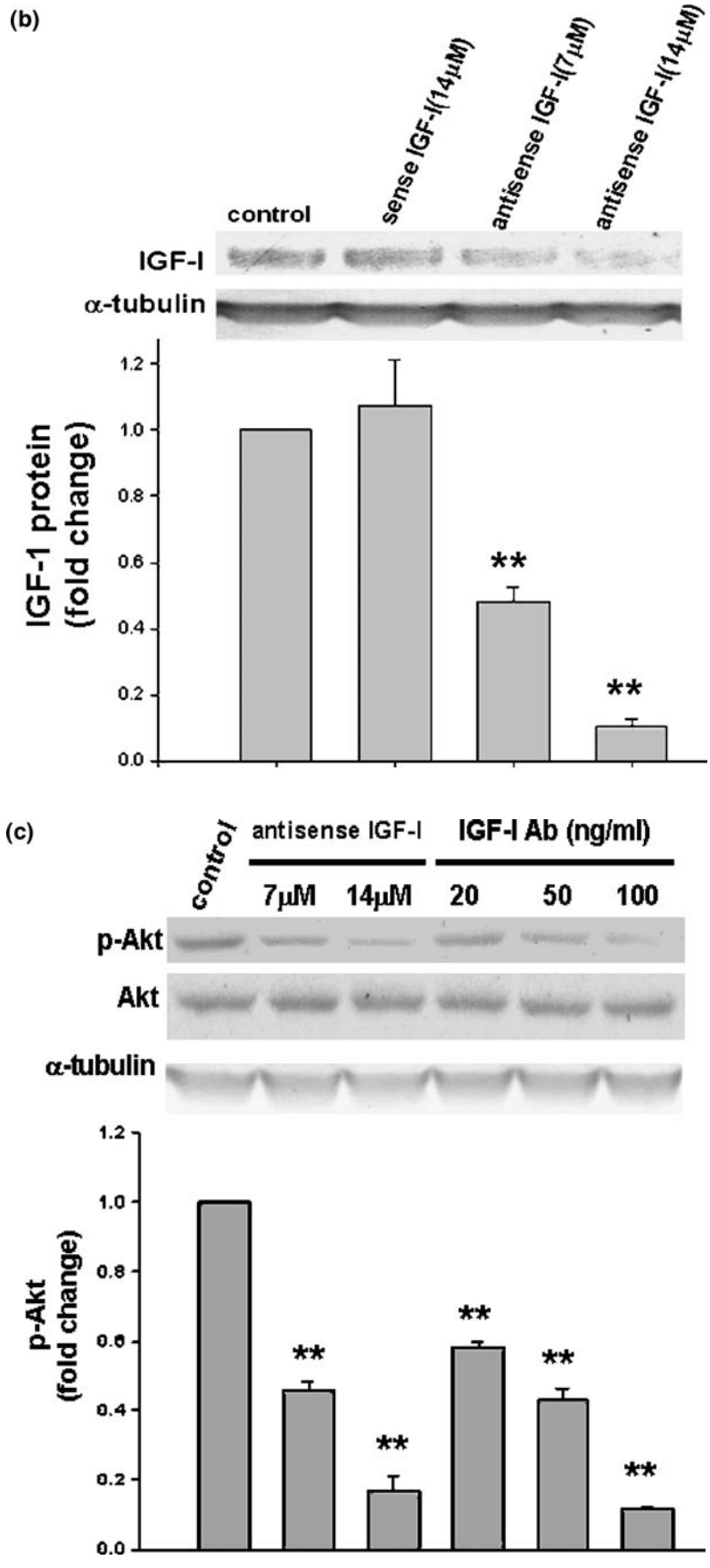
**Fig. 4** (Continued)



(Fig. 1). In addition, the strength of the synergistic effect on DNA fragmentation was dependent on the dosage of IGF-IR antibody (20, 40, and 60 ng/ml). The higher the concentrations of IGF-IR antibody, the more the DNA fragmentation (Fig. 1).

### Apoptosis measured by condensed nuclei with DAPI staining and TUNEL assay was synergistically induced by Ang II and IGF-IR antibody

To clarify whether cardiomyoblast apoptosis was induced by Ang II and/or IGF-IR antibody, DAPI staining and TUNEL assay were measured with or without administration of Ang II ($10^{-8}$ M) and/or IGF-I receptor antibody (100 ng/ml). The condensation of nuclei stained with DAPI, indicating cell apoptosis, was significantly ($P < 0.05$) increased by 3% after administration of Ang II ($10^{-8}$ M), and by 5% after administration of IGF-IR antibody (100 ng/ml) in H9c2 cells, compared to the negative control (Fig. 2). We observed a higher condensation of nuclei when Ang II ($10^{-8}$ M) and IGF-IR antibody (100 ng/ml) were combined, compared to the negative control (Fig. 2). This indicated that they exerted a synergistic rather than an additive effect on cell apoptosis.

### The amount of pro-apoptotic Bad protein and the release of cytochrome *c* were increased by Ang II and IGF-IR antibody

The clarify whether mitochondrial dependent apoptotic pathway was indused by Ang II and/or IGF-IR antibody, the protein product of Bcl2, Bad, and cytosolic cytochrome *c* were measured by Western Blotting with or without administration of Ang II ($10^{-8}$ M) and/or IGF-I receptor antibody (20, 40, 60 ng/ml). Western blot analysis revealed that the expression of Bad protein and the release of cytochrome *c* both increased in H9c2 after administration of either Ang II ($10^{-8}$ M) or IGF-IR antibody (20, 40, and 60 ng/ml), compared to negative and positive controls (Fig. 3). We observed a synergistic increase in production of Bad protein or release of cytochrome *c* when Ang II ($10^{-8}$ M) and IGF-IR antibody (20, 40, and 60 ng/ml) were combined (Fig. 3).

### IGF-IIR and IGF-II protein products were increased by IGF-I deficiencies or IGF-IR resistance

To clarify whether IGF-II, IGF-II receptor was up-regulated by IGF-I deficiencies or IGF-IR resistance, the protein product of IGF-II and IGF-II receptor were measured by Western Blotting with administration of antisense IGF-I, IGF-I antibody, IGF-I receptor antibody. Western blot analysis revealed that the IGF-IIR and IGF-II protein products were increased in H9c2 cells treated with antisense IGF-I (14 $\mu$M), IGF-IR antibody (100 ng/ml), or IGF-I antibody (100 ng/ml), whereas the IGF-IIR and IGF-II protein products were not affected by the treatment of sense IGF-I (14 $\mu$M) and non-specific IgG (a control antibody, 100 ng/ml) (Fig. 4(a)).

To confirm the effects of antisense IGFI on IGF-I, the protein product of IGF-I were measured after administration of antisense IGF-I. The amount of IGF-I protein products in H9c2 cells was not changed by sense IGF-I (14 $\mu$M) but was reduced by antisense IGF-I (7 $\mu$M and 14 $\mu$M) (Fig. 4(b)).

To clarify the activity of PI3 K-Akt pathway were affected by IGF-I deficiencies, the protein products of Akt were measured by Western Blotting with administration of antisense IGF-I, IGF-I antibody. The phosphorylated Akt protein products were significantly reduced by antisense IGF-I (7 $\mu$M and 14 $\mu$M) and IGF-I antibody (20, 50, 100 ng/ml) in a dose-dependent manner (Fig. 4(c)).

### Apoptosis induced by impaired IGF-I signaling or impaired IGF-I signaling plus Ang II was inhibited by antisense IGF-II or IGF-II antibody

To clarify whether cardiomyoblast apoptosis induced by impaired IGF-I signaling or impaired IGF-I signaling plus Ang II was inhibited by antisense IGF-II or IGF-II antibody, DAPI staining, TUNEL assay, and active caspase 9 and 3 were measured with or without administration of Ang II ($10^{-8}$ M), antisense IGF-I, IGF-I receptor antibody, antisense IGF-II, and/or IGF-II antibody. The condensation of nuclei stained with DAPI (Fig. 5(a)), TUNEL assay, (Fig. 5(b)), the activated forms of caspase 9 and caspase 3 (Fig. 5(c)), all of which indicating apoptosis, were significantly increased in cardiac H9c2 cells after administrations of antisense IGF-I (14 $\mu$M) only, after administration of antisense IGF-I plus Ang II ($10^{-8}$ M), and administration of IGF-IR antibody (100 ng/ml) plus Ang II ($10^{-8}$ M) (Fig. 5(a–c)). On the other side, the increased condensations of the same nuclei (Fig. 5(a)), the TUNEL positive cells (Fig. 5(b)), the increased activated forms of caspase 9 and caspase 3 (Fig. 5(c)), caused by administrations of antisense IGF-I (14 $\mu$M) only, administration of antisense IGF-I plus Ang II ($10^{-8}$ M), and administration of IGF-IR antibody (100 ng/ml) plus Ang II ($10^{-8}$ M) were significantly ($P < 0.05$) decreased after additional administration of antisense IGF-II (14 $\mu$M) and IGF-II antibody (100 ng/ml) in the cardiac H9c2 cells, respectively (Fig. 5(a–c)).

Bad protein products and the release of cytochrome *c* induced by Ang II plus IGF-I antibody or IGF-IR antibody were reduced by IGF-II antibody

To clarify whether synergistic cardiomyoblast apoptosis induced by angiotensin II plus impaired IGF-I signaling was regulated by IGF-II, the protein products of Bad and the release of cytochrome *c* were measured by Western Blotting with administration of Ang II ($10^{-8}$ M) plus antisense IGF–I and IGF-I antibody as well as with or without administration of IGF-II antibody. Western blot analysis showed that the amount of Bad protein and the release of cytochrome c were both increased in H9c2 after administration of either Ang II ($10^{-8}$ M) plus anti-sense IGF-I (14 $\mu$M) or after the administration of Ang II ($10^{-8}$ M) plus IGF-IR antibody (100 ng/ml) (Fig. 6). The increases in Bad protein products and in cytochrome *c* release induced by either Ang II ($10^{-8}$ M) plus anti-sense IGF-I (14 $\mu$M) or by Ang II ($10^{-8}$ M) plus IGF-IR antibody (100 ng/ml) were reduced by the administration of IGF-II antibody (100 ng/ml) (Fig. 6).

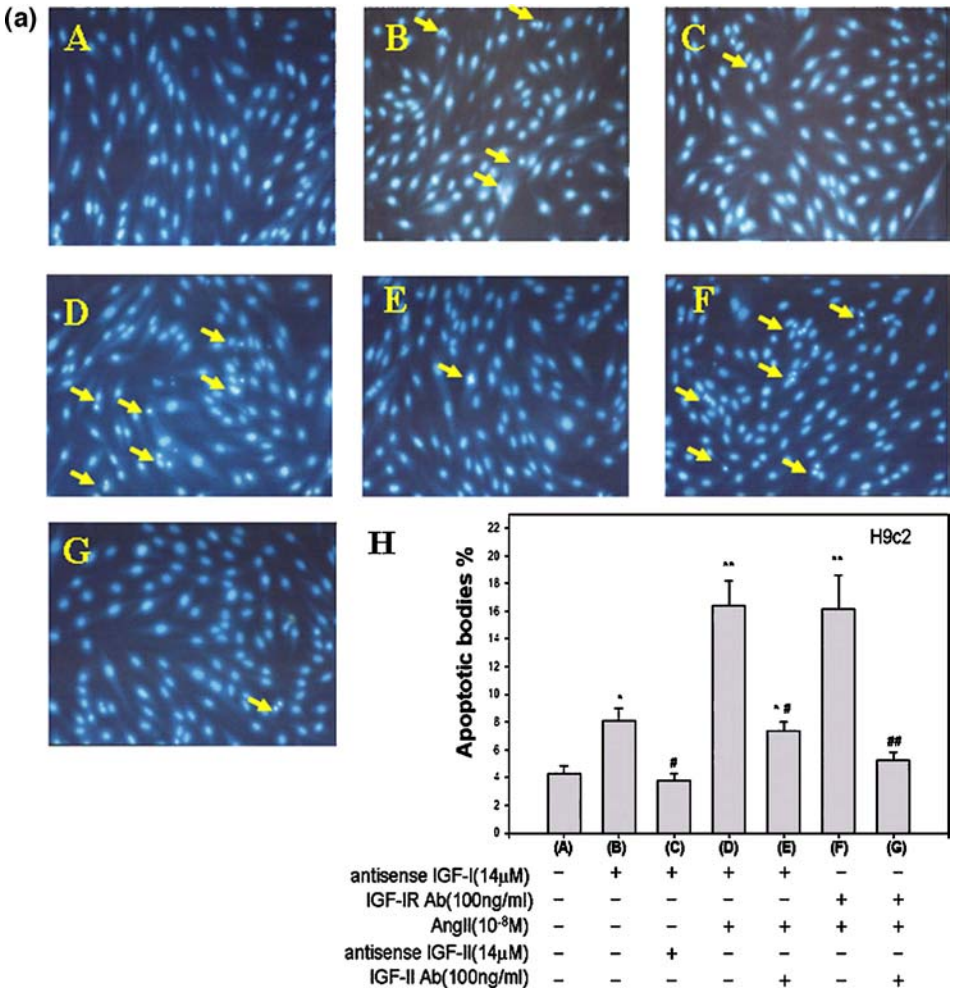
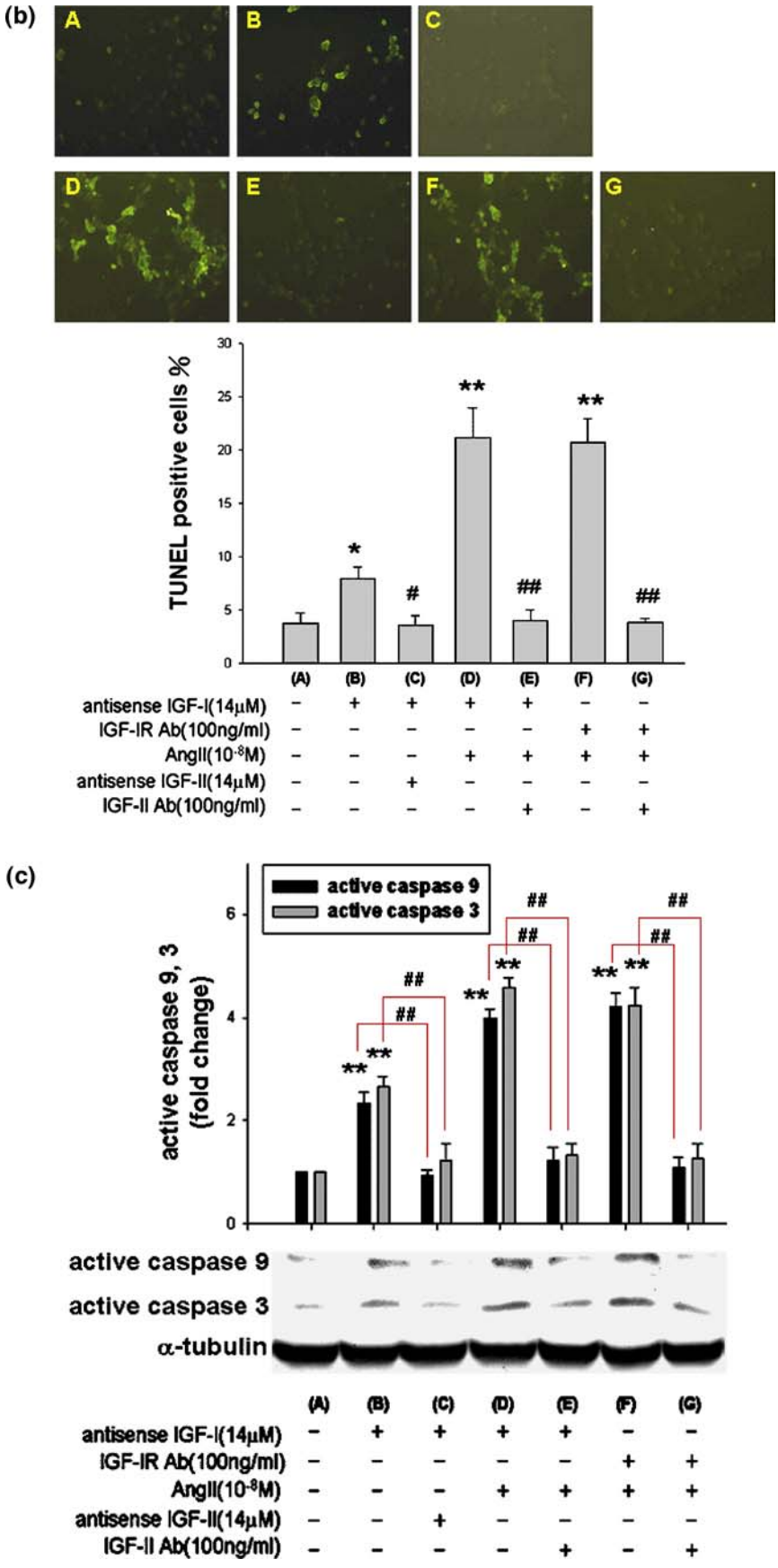



**Fig. 5** (a, b) Apoptosis induced by Ang II alone or plus antisense IGF-I and/or IGF-IR antibody was inhibited by antisense IGF-II and IGF-II antibody. H9c2 cells were treated with or without antisense IGF-I (14 $\mu$M), IGF-I receptor antibody (100 ng/ml), Ang II ($10^{-8}$ M), antisense IGF-II (14 $\mu$M), or IGF-II antibody (100 ng/ml). DAPI (4,6-diamidino-2-phenylindole) and TUNEL assay were determined. A, serum free; B, antisense IGF-I; C, antisense IGF-I plus antisense IGF-II; D, anti-sense IGF-I plus Ang II; E, antisense IGF-I plus Ang II plus IGF-II antibody; F, IGF-IR anti-body plus Ang II; G, IGF-IR antibody plus Ang II plus IGF-II antibody. The values are the mean $\pm$S.E. ($n = 3$, $n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$ represent significantly different when versus the cells treated with serum-free medium. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ represent the antisense IGF-II or IGF-II antibody effects. Arrow head indicated the nuclei condensed cells. (c) Pro-Apoptosis protein, activated forms of caspase 9 and 3 measured by Western Blotting were re-confirmed. Bars represent the relative quantification of activated forms of caspase 9 and caspase 3 protein products on the basis of control level and indicate mean fold change $\pm$ SE ($n = 4$). $^{**}P < 0.01$, significant differences from control group. $^{\#\#}P < 0.01$ significant effects of the antisense IGF-II or IGF-II antibody (Continue on next page)

**Fig. 5** (Continued)



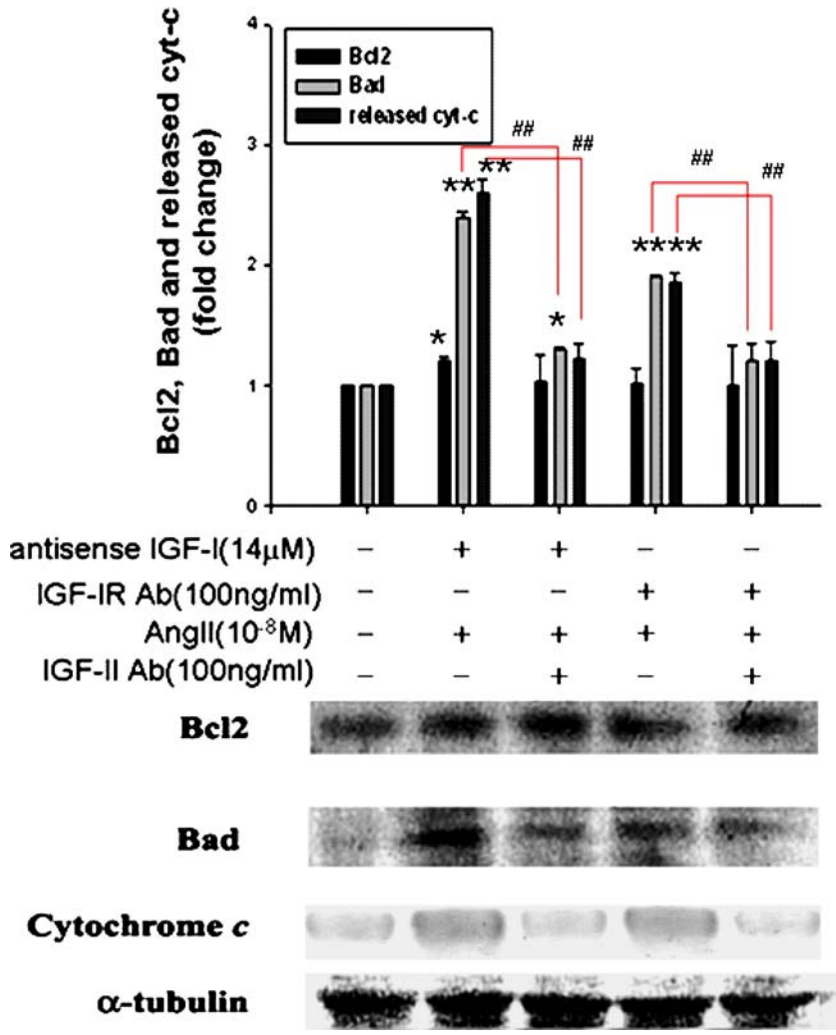



**Fig. 6** The increase of Bad protein products and cytochrome c release induced by antisense IGF-I plus Ang II or IGF-IR antibody plus Ang II, were reduced by IGF-II antibody treatment. H9c2 cells cultured in serum-free medium for overnight then treated with antisense IGF-I (14 $\mu$M) plus Ang II ($10^{-8}$ M), or IGF-I receptor antibody (100 ng/ml) plus Ang II ($10^{-8}$ M), with or without IGF-II antibody (100 ng/ml). The incubation was continued for another 24 h, and then the cells were harvested and extracted for the Western Blot analysis. Bars represent the relative quantification of Bcl2, Bad, and released cytochrome c protein products on the basis of control level and indicate mean fold change $\pm$ SE ($n = 4$). $^{**}P < 0.01$, significant differences from control group. $^{\#\#}P < 0.01$ significant effects of the IGF-II antibody

The synergistic DNA fragmentation induced by Ang II plus impaired IGF-I signaling was remarkably attenuated by the calcineurin inhibitor

To clarify whether cardiomyoblast apoptosis induced by angiotensin II plus impaired IGF-I signaling was regulated by calcineurin pathway, DNA fragmentation and active caspase 3 were measured with administration of Ang II plus antisense IGF-I and IGF-I antibody as well as with or without administration of calcineurin inhibitor (CsA and/or FK-506). H9c2 cardiomyoblast cells underwent DNA fragmentation and increase the active caspase 3 when exposed to either Ang II ($10^{-8}$ M) and antisense IGF-I (14 mM) or Ang II ($10^{-8}$ M) and IGF-IR antibody (100 ng/ml). The fragmentation was remarkably attenuated by the administration of CsA (1 $\mu$M) (Fig. 7(a) and (b)). A combination of IGF-IR antibody (100 ng/ml) and Ang II ($10^{-8}$ M) also had an effect on DNA fragmentation (Fig. 7(a)) and increase the active caspase 3 (Fig. 7(b)), which were attenuated by the administration of CsA (1 $\mu$M) (Fig. 7(a) and (b)) and FK-506 (Fig. 7(b)).

IGF-IR antibody plus Ang II or IGF-IR antibody plus IGF-II induced activated Bad protein products and the release of cytochrome *c* were remarkably attenuated by the CsA

To clarify the pathway from phosphorylated Bad to release of cytochrome *c* induced by IGF-I receptor resistance with and without angiotensin II was regulated by calcineurin pathway, the protein products of phosphorylated Bad, Bad, and cytosolic cytochrome *c* were measured. Western blot analysis showed that after the administration of IGF-IR antibody (100 ng/ml) plus Ang II ($10^{-8}$ M) or after the administration of IGF-IR antibody plus IGF-II (100 ng/ml) to H9c2 cells, there was a decrease in the amount of phosphorylated Bad protein and an increase in Bad protein and the released cytochrome *c*. These changes were remarkably attenuated by the administration of CsA(1 $\mu$M) (Fig. 8).

## Discussion

Our main findings can be summarized as follows: (1) By observing DNA fragmentation, stained cell nuclei and TUNEL assay in H9c2 cells, we found a synergistic effect on cardiomyoblast apoptotic process induced by IGF-I deficiency and/or IGF-IR resistance as well as Ang II (2); Western Blot analysis showed that the protein production of IGF-II and IGF-II receptor appeared to be upregulated by IGF-I deficiency and/or IGF-IR resistance in H9c2 cells, respectively (3); after blocking IGF-I and/or IGF-IR, the Ang II-induced increases in Bad pro-apoptotic protein and the release of cytochrome *c* were reduced by IGF-II antibody treatment; and [4] the apoptotic DNA fragmentation and active caspase 3 induced by Ang II plus IGF-IR antibody was attenuated by the calcineurin inhibitor, CsA and/or FK-506. After integrating our current findings into previously proposed apoptotic theory, our proposed hypothesis in cardiomyoblast apoptosis was presented in Fig. 9.

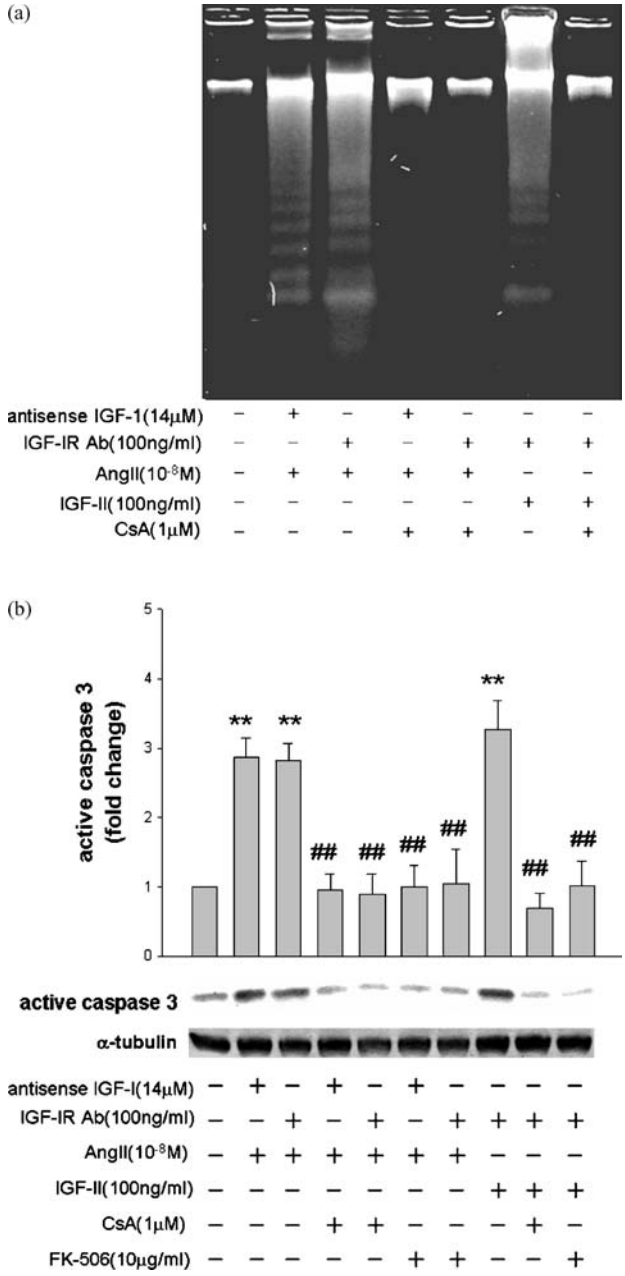



**Fig. 7** The synergistic DNA fragmentation and active caspase 3 induced by Ang II and IGF-IR antibody was remarkably attenuated by calcineurin inhibitor. H9c2 cells cultured in serum-free medium for overnight then treated with Ang II ($10^{-8}$ M) plus antisense IGF-I (14 $\mu$M), IGF-I receptor antibody (100 ng/ml), or IGF-II (100 ng/ml), with or without cyclosporine A (CsA, calcineurin inhibitor, 1 $\mu$M) and/or FK-506 (10 $\mu$/ml). The incubation was continued for another 24 h, and then the cells were harvested, extracted, and DNA fragmentation ($n = 3$) and active caspase 3 was determined ($n = 4$)

Angiotensin II has been reported to be involved in the stimulation of cardiac apoptosis in essential hypertension without major hemodynamic consequences [18, 19]. In the current study, when Ang II and IGF-I antibody or Ang II and IGF-IR antibody were co-administered, we observed a synergistic effect on cardiomyoblast apoptosis by the characteristic DNA fragmentation, DAPI stained cell nuclei, TUNEL positive cells, increased pro-apoptotic Bad, activated caspase 9, activated caspase 3 protein products, and the release of cytochrome *c*. This finding suggests that the stimulation of cardiac apoptosis will be exacerbated in hypertensive patients who have IGF-I deficiencies and/or IGF-IR resistances. IGF-I is an important survival growth factor that has been found to suppress myocardial apoptosis and exert an anti-apoptotic effect through the phosphatidylinositol 3-kinase/Akt kinase pathway and MAPK pathway in cardiomyoblast cells (Fig. 9) [8, 9]. Such findings indirectly support our findings that IGF-I deficiency or IGF-IR resistance, which block the IGF-I survival pathway, exacerbate cardiac apoptosis (Fig. 9). In addition, our data showed that higher concentrations of IGF-IR antibody caused more DNA fragmentation, Bad protein production, and cytochrome *c* release. These increases suggest the severity of angiotensin II-induced cardiomyoblast apoptosis is related to the severity of IGF-I deficiency and/or IGF-IR resistance. Our findings further support the previous concept that cardiomyoblast apoptotic levels are directly related to the amount of IGF-I deficiency and/or IGF-IR resistance [11].

The upregulation of IGF-II and IGF-II receptor in cardiomyoblast cells in this study appeared to be induced by IGF-I deficiency and/or IGF-IR resistance by treating with antisense IGF-I, IGF-IR antibody, or IGF-I antibody. One of our previous reports found a similar upregulation of IGF-II and IGF-IIR mRNA level in cultured adult rat ventricular cardiac cells after administration of IGF-I or IGF-IR antibody [13]. The IGF-II gene has been found to be expressed at high levels during embryonic and fetal life and at low levels in adult animals [20]. In one animal model used in an experiment on coronary artery occlusion, the short pulses of myocardial ischemia caused increased mRNA expression of

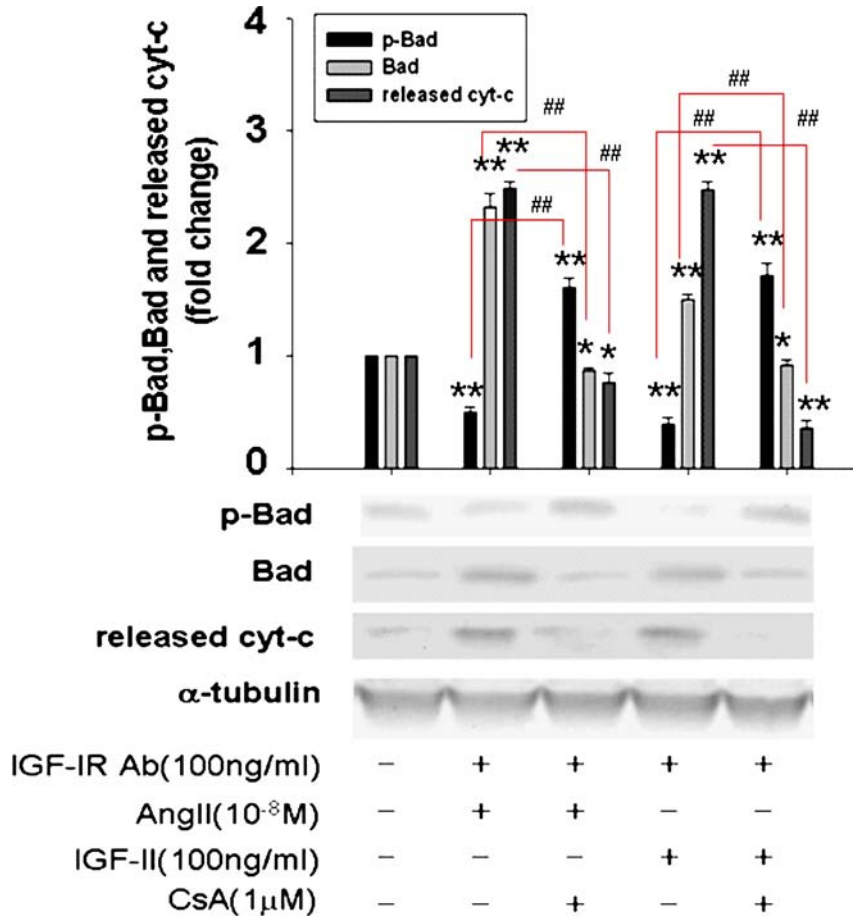



**Fig. 8** The synergistic apoptosis measured by phosphorylated Bad and Bad protein products and cytochrome c release induced by Ang II and IGF-IR antibody was remarkably attenuated by calcineurin inhibitor. H9c2 cells cultured in serum-free medium for overnight then treated with Ang II ($10^{-8}$ M), IGF-I receptor antibody (100 ng/ml), Ang II ($10^{-8}$ M) plus IGF-I receptor antibody (100 ng/ml), with or without cyclosporine A (CsA, calcineurin inhibitor, 1 $\mu$M). The incubation was continued for another 24 h, and then the cells were harvested, extracted, and phosphorylated Bad and Bad protein products and cytochrome *c* release was determined by Western Blotting. Bars represent the relative quantification of phosphorylated Bad, Bad, and released cytochrome *c* protein products on the basis of control level and indicate mean fold change $\pm$ SE ($n = 4$). $^{**}P < 0.01$, significant differences from control group. $^{\#\#}P < 0.01$ significant effects of CsA

the IGF-II gene [21]. The transactivation and over-expression of IGF-II gene in mice resulted in prenatal overgrowth and disproportionate organ overgrowth including cardiac abnormalities [22]. However, the mechanism of IGF-II and IGF-IIR regulated by IGF-I deficiency and/or IGF-IR resistance in cardiomyoblast cells is still unclear.

In this study, the increased condensation of nuclei stained, Bad, activated caspase 9, activated caspase 3 protein products, and the release of cytochrome *c* caused by impaired IGF-I signaling and/or angiotensin II were inhibited by the administration of antisense IGF-II and IGF-II antibody. The IGF-II may activate Gi-2, a GTP binding protein, activate a calcium-permeable cation channel through a cell surface IGF-II receptor, and further activate Bad pathway and Bad-related apoptosis [17, 23] (Fig. 9). IGF-II and IGF-II receptor appear to be up regulated by IGF-I deficiency and/or IGF-IR resistance and cardiomyoblast apoptosis induced by + IGF-I signaling appear to be restored by antisense IGF-II and IGF-II antibody. Therefore, we can speculate that IGF-I deficiency and/or IGF-IR resistance induces cardiomyoblast apoptosis and that the up regulation of IGF-II and IGF-IIR genes with their downstream pathway activation is augmented by Ang II (Fig. 9).

Since the synergistic effect of DNA fragmentation induced by Ang II plus IGF-IR antibody was remarkably attenuated by the calcineurin inhibitor, CsA, in the current study, we conclude that calcium ($Ca^{2+}$)-calcineurin is an important candidate pathway for the synergistic apoptotic effect of angiotensin II and IGF-I deficiency and/or IGF-IR resistance in cardiomyoblast cells. Angiotensin II is thought to activate calcium-calmodulin-dependent protein phosphatase calcineurin through Galphaq (G$\alpha$-q)/PLC signaling transduction, which is mediated by permeability transition pore formation and activation of the mitochondrial death pathway (Fig. 9) [16, 24]. Calcium-calcineurin might further dephosphorylate the transcription factor NFAT3, enabling it to translocate to the nucleus and interact with GATA transcription factor, resulting in synergistic activation of cardiac transcription and cardiac pathological hypertrophy [16, 25]. Cardiac pathological hypertrophy is rapidly succeeded by apoptotic cellular and molecular changes [24]. At the same time, $Ca^{2+}$/Calcineurin also dephosphorylates proapoptotic Bcl-2 family protein, Bad, and induces cytochrome *c* release from mitochondria to the cytosol, which further induces caspases activation, and cardiac cell apoptosis (Fig. 9) [17, 24, 25]. However, the IGF-II has been shown to directly

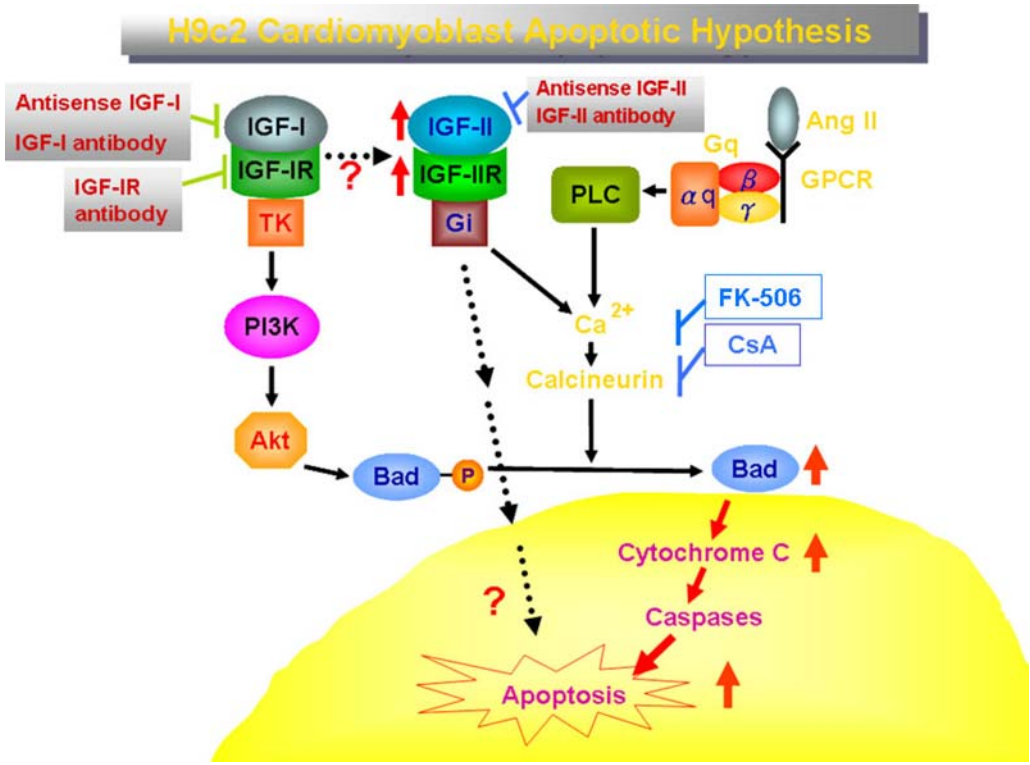



**Fig. 9** The proposed hypothesis of IGF-I, IGF-II, and Angiotensin II pathways in cardiomyoblast apoptosis relevant to this study. IGF-I binding IGF-I receptors via intracellular signaling pathways involving tyrosine kinase (TK) activity may exert an anti-apoptotic effect via phosphatidylinositol 3-kinase (PI3 K) and Akt kinase (Akt)-dependent Bad phosphorylation (Bad-p). Antisense IGF-I, IGF-I antibody, or IGF-I receptor antibody not only blocked IGF-I survival pathway, but also upregulated IGF-II and IGF-IIR (upper arrows) under IGF-IR resistance. Although the mechanism of upregulation of IGF-II system is still unclear, apoptosis induced by IGF-I resistance was inhibited by antisense IGF-II or IGF-II antibody. IGF-II may bind IGF-II receptors via Gi, a GTP binding protein and further activate a calcium ($Ca^{2+}$)-calcineurin-mediated dephosphorylation of Bad, which may further activate Bad-related apoptotic pathway. Angiotensin II (Ang II) was also considered to activate a calcium-calcineurin-mediated dephosphorylation of Bad through G-protein-coupled receptors (GPCR), Galphaq (Gα-q)/PLC signaling transduction. However, calcineurin inhibitor (cyclosporine A and FK-506) did attenuate the synergistic apoptosis induced by Ang II plus IGF-I resistance. Therefore, Ang II enhance IGF-IR resistant effect and exacerbate more severe cardiac apoptosis is somewhat mediated through the calcineurin activation. The dotting-lines represent unknown pathways

induce hypertrophy in adult rat cardiac cells in a serum–free medium as demonstrated by their increased size, total protein synthesis, and transcription of muscle-specific genes [26]. In addition to its activation of Gi-2 and calcium-calcineurin through a cell surface IGF-II receptor, IGF-II might, when the IGF-IR-dependent pathway is blocked, induce hypertrophy in adult rat cardiac cells via another two alternative pathways: an IGF-IR-dependent pathway or a lysosome-dependent pathway [27]. Primary ventricle myocytes culture can be used to confirm all these findings.

## Conclusion and possible clinical application

We found that IGF-I deficiency and/or IGF-IR resistance induced apoptosis in cardiomyoblast cells. The apoptosis, which might have been caused by the upregulation of IGF-II and IGF-IIR genes possibly activated the downstream calcineurin pathway, was synergistically augmented by Ang II. Our findings may raise a further question about whether myocardial cell death in patients with hypertension induced by Ang II and impaired IGF-I system is caused by an upregulation of IGF-II system and calcineurin activity. In diabetes, IGF-I receptor antibodies bind to the IGF-I receptor at or near the IGF-I binding sites, inhibit IGF-I binding, and probably cause IGF-I resistance [6]. Besides, the circulating IGF-I levels and IGF-I function fall progressively with aging [28]. Cardiovascular disease is reported to be a major cause of morbidity and mortality in patients with diabetes mellitus [29]. It has been suggested that specific vascular and myocardiopathic alterations are responsible for the excessive cardiovascular morbidity and mortality in diabetes [29]. Diabetic cardiac apoptosis may be viewed as an angiotensin II-dependent process, in which angiotensin II may play a critical role in myocyte death and hypertrophy [30]. The cardiovascular alterations in patients with diabetes mellitus manifest themselves clinically as hypertension, coronary heart disease, congestive heart failure and/or sudden cardiac death [29, 30]. In order to prevent cardiovascular

diseases in diabetes, it has been suggested that diabetic patients maintain excellent glycemic control, a normal blood pressure control, and be taking an angiotensin-converting enzyme inhibitor. Our findings may further suggest that diabetic patients maintain a normal IGF-I function and try to prevent the upregulation of IGF-II system, such as IGF-I supplement and/or IGF-II and calcineurin inhibitors. If this can be done, then cardiomyoblast apoptosis might be inhibited in diabetic patients with cardiovascular diseases. Further animal or human studies are required to confirm the possible signaling pathways and the clinical applications of this study.

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