Leu27IGF2 plays an opposite role to IGF1 to induce H9c2 cardiomyoblast cell apoptosis via Gαq signaling

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Abstract

This study examines the role of IGF2/mannose 6-phosphate receptor (IGF2R) signaling in the signaling transduction regulation and cell apoptosis in H9c2 cardiomyoblast cells. However, it is difficult to recognize the distinct activation of IGF2 signaling without interfacing with IGF1 receptor (IGF1R) after exposure to IGF2. Leu27IGF2, an analog of IGF2 that interacts selectively with the IGF2R, was used to specifically activate IGF2R signaling in this study. DNA fragmentation and TUNEL assay revealed that in contrast to IGF1 treatment preventing angiotensin II and AG1024-induced cell apoptosis, Leu27IGF2 appears to synergistically increase apoptosis in those cells. We further found cell apoptosis induction and an increase in the active form of caspase 3 in the treatment of cells with Leu27IGF2, but not IGF1. To detect the interaction between IGF2R and Gαq using the immunoprecipitation assay, we found that IGF2R could directly interact with Gαq and after treatment with Leu27IGF2 the binding ability of Gαq to IGF2R had increased. This sequentially resulted in the phosphorylation of phospholipase C-β, a key downstream modulator of Gαq, on serine 537. Moreover, disruption of the Gαq protein by small interferon RNA reduced the cell apoptosis induced by Leu27IGF2. Our findings demonstrate that IGF2R activation appears to induce cell apoptosis via Gαq-derived signaling cascades and its effect is completely different from IGF1R survival signaling.

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Introduction

Insulin-like growth factor II (IGF2) is able to interact with three different receptors: IGF1R, IGF2R, and the insulin receptor (IR) through distinct binding affinity. For instance, IGF2 has higher affinity to bind with IGF2R than IGF1R and IR respectively (Jones & Clemmons 1995). In general, after binding with IGF2, IGF2R stabilizes the local IGFs concentration through internalization and lysosomal degradation and is referred to as a ‘clearance receptor’ (Boker et al. 1997). Conversely, IGF2 to IGF1R or IR triggers a series of mitotic signaling cascades that function in cell development, growth, survival, and metabolic effect regulation (Jones & Clemmons 1995, Diaz et al. 2007, Blum & Baumrucker 2008). Recent evidence has shown that in spite of degrading IGF2 in the lysosome, IGF2R may spark intracellular signaling cascades in cell behavior regulation (Okamoto & Nishimoto 1991, McKinnon et al. 2001, Hawkes et al. 2006), but the function of IGF2R in signaling transduction in the heart remains unclear.

In a variety of cardiovascular disorders, including myocardial infarction, cardiomyocytes apoptosis in the heart has been identified as key to heart failure (Kang & Izumo 2000, Grazette & Rosenzweig 2005). Because the adult cardiomyocyte is not capable of cell proliferation, the prevention of cardiomyocyte apoptosis appears to
be a good strategy for prompting heart function in patients with heart failure (Fujio et al. 2000). In mammalian cells, cell apoptosis regulation in response to vast stresses is divided from either initiating the intrinsic or extrinsic pathway, thereby triggering caspases cascade activation (Riedl & Shi 2004). Studies of IGFs in a pressure overload animal model have explained physiological cardiac growth promotion, improved heart contractions, and attenuate cell apoptosis by activating the IGF1R survival signaling pathway. This has potential as a candidate being treated apoptosis by activating the IGF1R survival signaling pathway. This has potential as a candidate being treated for heart failure (Parrizas et al. 1997a, Ren et al. 1999, McMullen et al. 2004). However, the up-regulated protein expression of IGF2 in the heart undergoing hypertrophic, apoptotic, and ischemia stresses (Kluge et al. 1995, Huang et al. 2002, Kuo et al. 2006) has raised doubt about the role of IGF2 in the modulation of pathological situation biological responses.

In our previous study, we found that the upregulation of IGF2 and IGF2R genes is essential for angiotensin II (ANGII)-induced cell apoptosis and correlates with the promotion of cardiomyocytes apoptosis in hypertensive rat hearts (Lee et al. 2006). Based on this finding, we further propose that in addition to degrading IGF2, IGF2R is able to trigger the intracellular signaling cascades involved in cardiomyocyte apoptosis after IGF2 binding. However, it is difficult to study if the effect of IGF2 in controlling cell apoptosis is specifically via IGF2R. This study used Leu27IGF2, an analog of IGF2 that is specifically bound to IGF2R, but not IGF1R and InR (Beukers et al. 1991), to distinctly activate IGF2R signaling transduction. This led us to study its role in the regulation of cell apoptosis and compare its effect with that of IGF1 in H9c2 cardiomyoblast cells.

Our findings revealed that Leu27IGF2 specifically bound to IGF2R could trigger intracellular signals in a Gq-dependent manner, contributing to cell apoptosis, and played an opposite role to IGF1R in the survival signaling pathway. Inhibition of IGF2R signaling pathways may be a good strategy to prevent cell apoptosis and improve heart function.

Before further treatment with ANGII, IGF1, and Leu27IGF2, the cells were harvested overnight and then were extracted for analysis.

**Antibodies and reagents**

The following antibodies were used in this study: anti-IGF2R (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-caspase3 (Santa Cruz Biotechnology), anti-Akt (Santa Cruz Biotechnology), anti-phospho-Ser473-Akt (Cell Signaling Technology, Danvers, MA, USA), anti-Gzq (Santa Cruz Biotechnology), anti-Gzi (Santa Cruz Biotechnology), anti-Gz (Santa Cruz Biotechnology), anti-phospho-Ser537-phospholipase C-β (PLC-β) (Cell Signaling Technology), anti-PLC-β (Cell Signaling Technology), and anti-α-tubulin (Santa Cruz Biotechnology). All the secondary antibodies (anti-rabbit-HRP, anti-mouse-HRP, and anti-goat-HRP) and enhanced chemiluminescence were purchased from Santa Cruz Biotechnology. IGF1 was purchased from Sigma and Leu27IGF2 was obtained from GroPep (Adelaide, Australia). Insulin/IGF1R inhibitor AG1024 was obtained from BioSource International (Camarillo, CA, USA).

**Protein extraction and western blot analysis**

Cultured H9c2 cells were scraped and washed once with PBS. The cell suspension was then spun down and cell pellets 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 at 12,000 g for 10 min. The supernatants were then moved into new Eppendorf tubes for western blot analysis. Proteins from the H9c2 cell line were separated in 6–12% SDS-PAGE gradient and transferred to nitrocellulose membranes. Non-specific protein binding was blocked in blocking buffer at room temperature (RT) for 1 h (5% milk, 20 mM Tris–HCl, pH 7-6, 150 mM NaCl, and 0-1% Tween 20). The membranes were blotted with specific antibodies, as indicated in the figure legends, in blocking buffer at 4 °C overnight. Chemiluminescent detection was accomplished with Western Blotting Luminol Reagent (Santa Cruz Biotechnology). Densitometric analysis of immunoblots was performed using the Alphalamer 2200 digital imaging system (Digital Imaging System, San Leandro, CA, USA).

**TUNEL staining**

TUNEL staining was performed as previously described (Lee et al. 2006). After various treatments, H9c2 cells were fixed with 4% paraformaldehyde.
solution for 30 min at RT. After a rinse with PBS, the samples were incubated with a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and FITC-dUTP (Roche Applied Science) for 1 h at 37 °C using an apoptosis detection kit (Roche Applied Science). These cells were then stained with 4,6-diamidino-2-phenylindole to detect the cell nucleus using u.v. light microscopic observations (blue). The stained cells were examined in a drop of PBS under a fluorescence and u.v. light microscope (Olympus IX70 fluorescence microscope) at this state 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 $3 \times 10^5$ H9c2 cardiomyoblast cells. All morphometric measurements were performed using at least two individuals independently in a blind manner.

**DNA fragmentation**

After treatment, 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/ml RNase (Roche Molecular Biochemicals) for 2 h at 56 °C and 2.5 g/ml proteinase K (Roche Molecular Biochemicals) for 2 h at 37 °C, and only fragmented DNA was extracted. DNA was ethanol-precipitated and finally re-suspended in distilled water. The fragmented DNA was electrophoretically fractionated on 1.5% agarose gel and stained with ethidium bromide.

**Immunoprecipitation assay**

Immunoprecipitations (IPs) were performed from H9c2 cell lysates treated with IGF2 using a Catch and Release Reversible IP System (Billerica, MA, USA) according to the manufacturer’s instructions (Song et al. 2004). A total of 500 μg cell lysate was prepared and interacted with 4 μg of a specific primary antibody (IGF2R; Santa Cruz Biotechnology), 10 μl antibody capture affinity ligand, and enough 1× wash buffer was used to make up a final total volume of 500 μl, which was placed into a spin column and incubated on a rotator at 4 °C overnight. This ensured that the mixing-complex remained suspended during incubation. Immunoprecipitated proteins were eluted from the column with 1× denaturing elution buffer and separated by polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with antibodies (as indicated in the figure legends). Chemiluminescent detection was performed using western blotting luminol reagent (Santa Cruz Biotechnology).

**Small interferon RNA and transfection**

Double-stranded small interferon RNA (siRNA) sequences targeting guanine nucleotide binding protein zq polypeptide (Gzq; GenBank accession number NM_031036) mRNAs were obtained from Dharmacon (Lafayette, CO, USA). A non-specific duplex (5'-CAGUGGAGUAUACUGUGCAAGU-3'; Dharmacon), which did not significantly affect Gzq mRNA and protein levels relative to the untransfected controls, was used as a control. Concentrations of siRNA and the incubation intervals were tested. H9c2 cardiomyoblast cells were plated in 24-well or 100 mm well plates in DMEM without fetal bovine serum and transfected with double-stranded siRNA using the DharmaFECT Duo Transfection Reagent (Dharmacon) according to the manufacturer’s instructions. To assess gene silencing, Gzq protein levels were detected by immunoblotting.

**Densitometry and statistical analysis**

The protein-related intensities were analyzed using the AlphaImager 2200 digital imaging system (Digital Imaging System). In addition, all values were normalized to their respective lane load controls. All data presented in the text and figures as means ± S.E.M. The percentage of TUNEL-positive cardiac myocytes (Fig. 1d) was analyzed using one-way ANOVA with preplanned contrast comparisons against the control group (serum free) or against the Leu27IGF2 group. Results in Figs 2c, 3b, 3d, 4b, 4d, 4f, 4h and 1b were analyzed using unpaired Student’s t-test. In all cases, P values < 0.05 were considered significant.

**Results**

**Leu27IGF2 has opposite effects of IGF1 on regulating cell apoptosis**

In our previous study, the inhibition of IGF2 and IGF2R protein rescued cell apoptosis induced by ANGII (Lee et al. 2006). We implied that specific binding of IGF2 to IGF2R might play a critical role in cell apoptosis regulation in H9c2 cardiomyoblast cells. We used Leu27IGF2, an analog of IGF2 that distinctly binds to IGF2R (Beukers et al. 1991), to study the role of IGF2R signals in regulation of the ANGII-induced cell apoptosis. We compared its effect with that of IGF1. DNA fragmentation assay was performed to detect cell apoptosis. We found that treatment with IGF1, but not Leu27IGF2, attenuated the ANGII-induced DNA fragmentation (Fig. 2a). Additionally, our previous study on IGF1R resistance exhibited that the upregulation of IGF2 is needed for cell apoptosis induction using an
IGF1R resistant (Kuo et al. 2006). Therefore, to inhibit IGF1R-survival signals using AG1024 (Parrizas et al. 1997b), TUNEL assay revealed that 7% increase in cell apoptosis occurred in the IGF1R activity blockage using AG1024 compared with the untreated control (Fig. 2b and c). We further found 2.5-fold more apoptotic cells in H9c2 cardiomyoblast treated with Leu27IGF2 along with AG1024 than treated with AG1024 alone. Treatment with IGF1 significantly reduced the number of apoptotic cells induced by AG1024 (Fig. 2b and c).

**Leu27IGF2 induces caspase 3 activity and cell apoptosis in H9c2 cardiomyoblast**

We investigated whether the treatment with Leu27IGF2 would directly increase the active form of caspase 3 and cell apoptosis induction in H9c2 cardiomyoblast cells. Western blots revealed that the treatment with IGF1, but not with Leu27IGF2, increased the phosphorylation of Akt at 30 min, when compared with the untreated control (Fig. 3a and b), confirming that Leu27IGF2 does not activate IGF1R. Otherwise, the active form of caspase 3 was detected only in cells treated with Leu27IGF2 (Fig. 3a–d). Our findings further revealed that the treatment with Leu27IGF2 increased the active form of caspase 3 in time- and dose-dependent manners (Fig. 3a–d). Using TUNEL stain to detect cell apoptosis, we further found that there was ~11-fold increase in the apoptotic cells in cells treated with Leu27IGF2 (10^{-8} M)-induced cell apoptosis (upper panels). These cells were stained with DAPI (blue) to mark cellular nuclei. (d) The percentage of positive apoptotic cells is based on the percentages calculated for three sections each. Data were presented as means ± S.E.M. Bars indicate averages, *P < 0.05 values based on comparisons with untreated controls; #P < 0.05 values based on comparisons with treated Leu27IGF2. Full colour version of this figure available via http://dx.doi.org/10.1677/JME-08-0121.

**Leu27IGF2 regulates the Gα-derived signaling via interacting IGF2R with Gα protein**

It has recently been found that there is a putative G-protein binding site within the IGF2R cytoplasmic domain (Ikezu et al. 1995) and that IGF2 binding with IGF2R activates a G-protein sensitive-dependent pathway through which a variety of physiological
functions are triggered (Okamoto & Nishimoto 1991, McKinnon et al. 2001, Hawkes et al. 2006). Using the IP assay to determine whether IGF2R directly interacts with the Gaq proteins, we found that treatment with Leu27IGF2 at 30 and 60 min could enhance the IGF2R interaction with Gaq (Fig. 4a and b), have a dose-dependent manner (Fig. 4c and d). Furthermore, western blots revealed that serine 537 of PLC-β3 was phosphorylated within 30 and 60 min of Leu27IGF2 treatment (Fig. 4e and f) and an increase in PLC-β3 phosphorylation by Leu27IGF2 with a variety of concentration was followed in dose-dependent manner (Fig. 4g and h). Taken together, our results suggested that by regulating the interaction between IGF2R with Gaq proteins, Leu27IGF2 might activate the downstream modulators of the Gaq signaling pathway.

**Leu27IGF2 induces cell apoptosis through Gaq**

We further investigated whether Gaq may be involved in Leu27IGF2-induced cell apoptosis by using Gaq siRNA to disrupt Gaq protein expression in H9c2 cardiomyoblast cells. Western blots showed that there was a significant decrease in the Gaq protein level in H9c2 cardiomyoblast cells transfected with Gaq siRNA compared with cells transfected with non-specific siRNA (Fig. 1a and b). As shown in Fig. 1c and d, we found that Gaq protein expression inhibition by siRNA significantly reduced Leu27IGF2-induced cell apoptosis. These findings indicated that the Gaq protein is required for Leu27IGF2-induced cell apoptosis.

**Discussion**

We used Leu27IGF2 to ensure specific IGF2R activation and compared its effect with IGF1 on cell apoptosis regulation in H9c2 cardiomyoblast cells. Our present data indicated that by regulating Gaq and IGF2R interaction, Leu27IGF2 triggered G protein-coupled receptor-like intracellular signaling pathways that were completely different from the IGF1-derived cell survival signaling pathway. A depiction of this process is shown in Fig. 5.

Although IGF1 and IGF2 have highly homologous protein structures and play similar roles in cell growth and development, they have been reported to act...
Figure 3 Leu27IGF2 induces H9c2 cardiomyoblast cell apoptosis. (a) H9c2 cardiomyoblast cells were left untreated (0 h) or were stimulated with IGF1 (10^{-8} M), and Leu27IGF2 (10^{-8} M) respectively for the indicated times. Protein lysates were prepared and immunoblotted assays were blotted with the indicated antibodies. Treatment with IGF1 increased the phosphorylation of Akt in a time-dependent manner, whereas the treatment with Leu27IGF2 was not able to phosphate Akt protein. However, the induction of an active form of caspase 3 protein was only detected in the Leu27IGF2 treated cells. (b) Data were quantified using densitometry and expressed as a fold change in the untreated control. Results are shown as mean ± S.E.M of three independent experiments performed in duplicate. Statistical significance: *P<0.05; **P<0.01, Leu27IGF2 treated versus untreated controls. (c) Western blots analysis of H9c2 cardiomyoblast cells treated with Leu27IGF2 (10^{-9}, 10^{-8}, and 10^{-7} M) in 24 h using specific antibodies as indicated. α-Tubulin served as a loading control. (d) Data were quantified using densitometry and expressed as a fold change in the untreated control. Results are shown as mean ± S.E.M of three independent experiments performed in duplicate. Statistical significance: *P<0.05; **P<0.01, Leu27IGF2 treated versus untreated controls. (e) TUNEL staining of H9c2 cardiomyoblast treated with IGF1 (10^{-8} M) and Leu27IGF2 (10^{-8} M) respectively, for 24 h. Number of positive apoptotic cells ~12-fold increased in the presence of Leu27IGF2 compared with the untreated control (upper panels). Treatment with IGF1 cannot induce cell apoptosis. DAPI (blue) was used to mark nuclei (lower panels). (f) The percentage of positive apoptotic cells is based on the percentages calculated for three sections for each treatment. An excess of 300 cells were involved in the cell death count and quantification. Data were presented as means ± S.E.M. Bars indicate averages, *P<0.05. Full colour version of this figure available via http://dx.doi.org/10.1677/JME-08-0121.
Figure 4 Leu27IGF2 regulates the interaction of IGF2R with Gα protein and activates PLC-β. Protein lysates from H9c2 cardiomyoblasts treated with Leu27IGF2 (10⁻⁸ M) for the indicated time were subjected to immunoprecipitation analysis using anti-IGF2R antibody and following immunoblot assay with anti-Gαq antibody to show the interaction between IGF2R and Gαq. The Gαq protein was found to have increased ability to associate with IGF2R in the presence of Leu27IGF2. α-Tubulin served as a load control. (b) Data were quantified using densitometry and expressed as a fold change in the untreated control. Results are shown as mean ± S.E.M. of three independent experiments performed in duplicate. Statistical significance: *P<0.05, LeuIGF2 treated versus untreated controls. (c) Protein lysates from H9c2 cardiomyoblasts treated with Leu27IGF2 (10⁻⁸ M) for 1 h. Immunoprecipitation assay was performed to detect the interaction between IGF2R and Gαq using anti-IGF2R and followed by immunoblot assay with anti-Gαq antibody. (d) Data were quantified by densitometry and presented as mean ± S.E.M. Statistical significance: *P<0.05; #P<0.01, Leu27IGF2 treated versus untreated controls. (e) After treatment with Leu27IGF2 (10⁻⁸ M), western blot analysis applied to detect the phosphorylation of PLC-β3 at serine 537 residue. There was an increase in PLC-β3 phosphorylation in the cells treated with Leu27IGF2 in a time-dependent manner. (f) Data were quantified using densitometry and expressed as a fold change in the untreated control. Results are shown as mean ± S.E.M. of three independent experiments performed in duplicate. Statistical significance: *P<0.05; #P<0.01, Leu27IGF2 treated versus untreated controls. (g) Western blot analyses of cell lysates of H9c2 cardiomyoblast cells treated with Leu27IGF2 (10⁻⁸ M) for the 1 h using anti-phospho-PLC-β3 antibody. (h) Data were quantified by densitometry and are presented as mean ± S.E.M. Statistical significance: *P<0.05; #P<0.01, Leu27IGF2 treated versus untreated controls. n=3 independent experiments for each data point.
physiological functions. In addition, creating a specific blocker for inhibiting IGF2R signaling or enhancing the IGF1R survival signal may be a good idea in the prevention of cardiac hypertrophy and cardiomyocytes apoptosis.

Several investigations have shown that IGF2R interacts with the Gz protein to affect cell behaviors by activating specific intracellular signaling cascades (Okamoto & Nishimoto 1991, McKinnon et al. 2001, Hawkes et al. 2006). Conversely, in mouse L-cell membranes and phospholipid vesicles, IGF2R is not able to interact with a small G-protein (Korner et al. 1995). In this study, our IP assay of H9c2 cardiomyoblast cells that were derived from embryonic rat heart tissue demonstrated that IGF2R directly interacted with the Gzq protein (Fig. 4a–d), suggesting that this interaction and sequentially IGF2R signaling activation may occur in a tissue-specific manner. It is very interesting to investigate whether IGF2R signaling activation exists in human hearts with heart disease in the future.

Our finding further revealed that an increase in the binding ability of IGF2R and Gzq after treatment with Leu27IGF2 may result in the phosphorylation of PLC-β3 on serine 537 (Fig. 4e–h). We propose that Leu27IGF2 binding to IGF2R might change its conformation to attract different effectors like GEF creating multiple protein complexes (Rockman et al. 2002). These complexes promote Gzq binding to GTP, resulting in their dissociation from the Gβγ subunits. In response to extracellular stimuli, the activation of PLC-β3 by an α-subunit of heterotrimeric G-proteins thereby hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate two secondary messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol, contributing to trigger a calcium influx and intracellular signaling cascades such as PKC, calcineurin, and CaMKII (Smrcka et al. 1991, Singer et al. 1997). In the following, phosphate serine 537 of PLC-β3 by CaMKII could restore PLC-β3 activity to the basal level (Yue & Sanborn 2001), suggesting that the treatment with Leu27IGF2 might enhance calcium influx and subsequently activate CaMKII. In addition, a calcium-sense protein phosphatase, calcineurin, has been identified as a cell apoptotic inducer involved in the Gzq signaling to impair major outer membrane protein (MOMP) and activate caspase 3 by dephosphorylation of Bad (Balke & Shorofsky 1998, Wang et al. 1999). Although our findings observed that the Gzq protein is required for Leu27IGF2-induced cell apoptosis (Fig. 1), the detailed cell apoptosis regulation mechanism via IGF2R will need further investigation.

In conclusion, the results of this study explained that Leu27IGF2 and IGF1 act differently in the control of cell apoptosis. Treatment with Leu27IGF2 specifically binds to and activates IGF2R to trigger differently in the regulation of endogenous acetylcholine release and fetal growth promotion (Kar et al. 1997, Sferruzzi-Perri et al. 2006). We further observed that the existence of IGF2R signaling cascades in H9c2 cardiomyoblast cells was activated in the treatment of Leu27IGF2, than IGF1, providing a new insight to differentiate between IGF1 and IGF2 biological functions.

Studies of IGF1 deficiency and/or IGF1R resistance in cardiomyocytes exhibit that upregulation of IGF2 is essential to causing cardiac hypertrophy and apoptosis (Huang et al. 2002, Kuo et al. 2006). Consistent with our data, the IGF1 promotes cell survival via activating IGF1R signaling and also inhibits cell apoptosis by downregulation of IGF2R signaling (Fig. 2). We propose that a whole IGF system in the heart might play a balancing role in cell apoptosis and improve cell survival by activating IGF2R and IGF1R (Fig. 3). In further research, it would be interesting to investigate if IGF2R signaling is involved in the regulation of various
intracellular signals via regulation of the interaction of IGF2R and the Gαq protein, which participate in cell apoptosis. Our findings gained from H9c2 cardiomyoblast cells specifically suppressing IGF2R signaling might be applied potentially to prevent heart failure progression with cardiomyocytes apoptosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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