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行政院國家科學委員會補助專題研究計畫成果報告

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※Antisense oligodeoxynucleotide targeted to insulin-like ※
※growth factors I and II as a novel strategy to investigate ※
※cardiac hypertrophy ※
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計畫主持人：黃志揚 助教授

共同主持人：劉哲育 副教授

李怡靜 副教授

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：私立中山醫學院生化所

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行政院國家科學委員會專題研究計畫成果報告

Preparation of NSC Project Reports

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一、中文摘要

已知昇血壓素-II（Ang-II）、類胰島素生長因 I 及 II（IGF-I, II），均與心肌肥大有直接相關，且可透過內分泌、自主分泌及側分泌的方式來調控心肌細胞。由於反向寡去氧核酸序列（ODN）法可專一性的與 mRNA 黏合，進而抑制 RNA 的轉譯及基因產物的形成。

我們乃使用 IGF-I 及 IGF-II 的反向 ODN 來標的他們相關 mRNA，藉由抑制他們 mRNA 的轉譯，來探討在引發心肌肥大的過程中，IGF-I、IGF-II 及 Ang-II 之間的交互作用關係。將成鼠心室心肌細胞分離並培養於血清的培養液中，加或不加 Ang-II、反向 IGF I-ODN 及反向 IGF II-ODN。我們的結果指出，心肌細胞在 10^{-8} μM 的 Ang-II 培養下，24 小時漲大了 24%，48 小時漲大了 55%，而不同濃度的反向 IGF I-ODN 均可誘發心肌細胞肥大，而即使僅以 1.38μM

反向 IGF I-ODN 同時加入 10^{-8}μM 的 Ang-II 卻可造成心肌細胞 210%的漲大。有趣的是，此種現加成性的心肌肥大誘導，發生於 10^{-8}μM 的 Ang-II 及不同濃度的反向 IGFI-ODN 刺激。然而，當 10^{-8}μM 的 Ang-II 及不同濃度的反向 IGFI-ODN，或單獨以反向 IGFII-ODN 加入心肌細胞，卻不見心肌肥大現象產生。我們首次的揭發了 IGF-II 比 IGF-I 及 Ang-II 更直接的調控心肌細胞肥大。

關鍵詞：專題計畫、報告格式、國科會

Abstract

Angiotensin II(Ang II) and insulin-like growth factor I and II(IGF-I and IGF-II) are associated with cardiac hypertrophy in an endocrine, paracrine, and/or autocrine manner. Antisense oligodeoxynucleotides(ODNs) provide a novel strategy to inhibit RNA transcription and the synthesis of the gene product. Because antisense ODNs hybridize with the mRNA strand, they are highly specific. To determine how Ang II, IGF-I and IGF-II might act/interact to cause hypertrophy, we used antisense IGF-I and IGF-II to target their respective mRNA. Adult rat ventricular cardiomyocytes were isolated in serum-free medium, with or without the addition of Ang II, antisense IGF-I, and antisense IGF-II. Our studies indicate that cultured ventricular cardiomyocytes hypertrophied by 24% at 1d and 55% 2d with exposure to Ang II(10-8M). Antisense IGF-I(at varying concentrations) by itself also initiated hypertrophy, where a maximum of 210% increase was observed at 1.38uM. Interesting, the addition of both Ang II(10-8M) and antisense IGF-I(at varying concentrations) resulted in an additive hypertrophy of culture cardiomyocytes. However, the addition of Ang II (10-8M) and antisense IGF-II(at varying concentrations), as well as antisense IGF-II(at varying concentrations) by itselfs, completely inhibited the hypertrophic response. We have revealed for the first time that IGF-II is more directly involved in regulating cardiac hypertrophy than Ang II or IGF-I.

Keywords:

Antisense oligodeoxynucleotide,

insulin-like growth factors I and II, cardiac hypertrophy.

二、緣由與目的

Cardiac hypertrophy is an independent indicator of cardiac morbidity and mortality in valvular disease, cardiomyopathy, hypertension, and ischemic heart disease (Thurman et al., 1998; Wong et al., 1997). The primary characteristics of hypertrophy include increases in mass, volume, contractile protein content and gene expression, and expression of embryonic genes (Foncea, 1997). The short-term mechanical stimulus to hypertrophy is well comprehended. However, the long-term response to mechanical stress and the biochemical factors that orchestrate the hypertrophic response remain largely unresolved.

Humoral factors, such as Ang-II, IGF-I, and IGF-II, have been demonstrated to influence the cardiomyocyte response to hypertrophy in many studies (Morgan et al.,1998). Ang II, IGF-I, and IGF-II elicit a wide variety of physiological effects on growth. Therefore, they may perform vital hormonal and/or autocrine/paracrine functions in the cardiomyocyte that allow them to participate in the hypertrophic response. However, all their roles in cardiovascular physiology and function as well as how they might act/interact to produce cardiac hypertrophy are not known.

The antisense ODNs strategy is useful research tool capable of elucidating the specific action/interaction of distinct factors and the molecular events that lead to cardiac hypertrophy. Antisense ODNs are highly specific because they hybridize with target mRNA strands, thus inhibiting translation and protein expression. We utilized antisense ONDs of IGF-I and IGF-II in culture, to provide a controlled setting in which we can further investigate the molecular events that transpire during cardiac hypertrophy and suggest pathways that may operate *in vivo*. By specifically targeting antisense ODNs to IGF-I and IGF-II mRNA, we can determine that direct contributions of IGF-I and IGF-II to cardiac hypertrophy, and the manner in which they might act/interact with Ang II.

三、研究報告應含的內容

Results:

Adult ventricular cardiomyocytes were exposed to either no treatment, Ang II (10^{-8}M) and/or varying concentrations of antisense IGF-I and antisense IGF-II for 2 days. Ang II(10^{-8}μM) was chosen among other concentrations (10^{-9}μM to 10^{-6}μM) from previous experiments performed in this lab by Huang and Buetow. Although hypertrophy occurs at 10^{-8}μM to 10^{-6}μM Ang II, 10^{-8}μM Ang II is ideal since it is approximately equal to the physiological level. Cardiomyocytes after treatment with Ang II (10^{-8}μM) apparently became enlarged by 20% at 24h (1.156 ± 18 μm^2) and 50 % at 48 h (1.409 ± 21μm^2) compared to that of non-treated cells at 24 h (908 ± 3 μm^2) and 48 h (912 ± 7 μm^2). Increase in cell surface area was significantly ($p<0.01$).

The antisense ODNs exhibited a concentration-dependent behavior. Fig. 2 shows dose-response curves examining cardiomyocyte hypertrophy following a 24 h and 48 h incubation in serum–free media containing 1.38 μM, 6.90 μM, and 13.80 μM concentrations of antisense IGF-I. Antisense IGF-I stimulated hypertrophy occurred in a concentration-dependent manner (1.38 μ M to 13.80 μM) ; amaximum effect (210% increase) was observed at 1.38 μM. The addition of Ang II (10^{-8} μM) to antisense IGF-I (6.90 μ M) resulted in statistically significant ($p<0.01$) additive hypertrophic behavior (1889 ± 7μm^2). Thus, significant cardiac hypertrophy could be induced by antisense IGF-I *in vitro* in adult rat cardiomyocytes.

The effect of incremental doses of antisense IGF-II (0.75μM to 18.85 μM) in cultured cardiomyocytes is shown in Figure 3. All antisense IGF-II treatment concentrations were associated with no significant difference compared with non-treated controls at 1 d and 2 d.Similarly, the addition of Ang II (10^{-8}M) to antisense IGF-II resulted in no significant difference compared with non-treated controls

at 1 d and 2 d. These findings suggest that antisense IGF-II cannot induce hypertrophy in adult rat cardiomyocytes.

Discussion:

This study was designed to assess the effects of antisense IGF-I and antisense IGF-II *in vitro*, in order to determine which factor, Ang II, IGF-I, or IGF-II, is specifically involved in cardiac hypertrophy. The results demonstrate that the antisense ODNs complemntary to the first fifteen codons of IGF-I and IGF-II exert specific inhibition of their function. An antisense ODN against rat IGF-I and IGF-II mRNA specifically induces the degradation of endogenous IGF-I and IGF-II mRNA. Our present findings in the cultured adult rat cardiomyocyte model utilizing an antisense approach provide new evidence that IGF-II is specifically associated with the activation of a hypertrophic response.

Our results are consistent with previous studies, which reveal that the activation of AT_1 receptors by increased levels of Ang II stimulates hypertrophy in animal and human cardiomyocytes (Sabri et al., 1998; Liu et al., 1998). However, a study by Harada et al. (1998) divulged that Ang II is not the most important factor in cardiac hypertrophy. Ang receptor subtypl (AT_1) mediates all the physiological actions of Ang II (Thurman, 1998). Yet, an $AT_{1\text{-knockout}}$ mouse model did not prevent development of cardiac hypertrophy (Harada et al., 1998). Therefore, an alternate signaling mechanism exists for the development of hypertrophy (Harada et al., 1998).

A study by Duerr et al. (1995) reveals the capacity of IGF-I to induce *hypertrophy in vivo*. IGF-I activates multiple signal transduction pathways, which may be responsible for hypertrophy (Foncea, 1997). However, we have been able to show that IGF-I is not necessary to elicit the hypertrophic response in cardiomyocytes. When antisense IGF-I were added to cultured cardiomyocytes, the cells were able hypertrophy despite the specific down-regulation of IGF-I mRNA. Therefore, certain factors can compensate for IGF-I when it is inhibited.

We believe that inhibition of the IGF-I mRNA results in the upregulation of IGF-II, which directly activates the hypertrophic response in cultured cardiomyocyte systems. In recently completed but unpublished studies in our lab, Huang and Buetow have revealed that the addition of IGF-I antibodies to the IGF-I receptor results in an increase in IGF-II and IGF-II receptors locally, resulting in adult rat cardiomyocyte hypertrophy. IGF-II mediates signal transduction leading to metabolic responses by binding to cell-surface IGF-I receptors (Decker et al., 1995; Liu et al., 1996).

That IGF-II can induce hypertrophy when the actions of IGF-I are inhibited, is consistent with the data obtained by this study, where the additionof antisense IGF-I in cultured cardiomyocytes and subsequent specific inhibition of IGF-I mRNA also resulted in cardiac hypertrophy. Thus, our results suggest that IGF-II can compensate for IGF-I, when it is deficient in the cardiomyocyte. Furthermore, IGF-II produces a more direct effect on cultured adult rat cardiomyocytes, because IGF-I alone cannot stimulate cardiac hypertrophy when IGF-II mRNA is inhibited. Finally, the addition of Ang II and antisense IGF-I produced an additive hypertrophic behavior, which suggests that IGF-II expression was increased to compensate for IGF-I deficiency in the cardiomyocyte.

The application of antisense ONDs in controlling the development of cardiac hypertrophy in culture is a novel strategy. Specifically abolishing the activity of IGF-I and IGF-II in the cardiomyocyte enabled us to determine that IGF-II is more directly involved in regulating cardiac hypertrophy, than IGF-I or Ang II. However, because of species differences in the pathways of Ang II and antisense-IGF I, and IGF-II formation between human and rat, our results on the stimulation or prevention of hypertrophy in rats are not

conclusive in humans. In future studies, we intend to measure the level of IGF-II mRNA in antisense IGF-I inhibited cardiomyocytes to delineate that IGF-II compensates for IGF-I deficiency in cardiac hypertrophy.

四、計畫成果自評

實驗進行順利，成果顯著。

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Addition	Experiment I			
	Day 1		Day 2	
	Size (μM2)	Percent of Control	Size (μM2)	Percent of Control
None (Control)	910.153	100.0	919.361	100.0
Ang II (10^{-8} M)	1145.75**	125.9	1434.309**	156.0
Ang II (10^{-8} M) + 1.38 μM A-IGF-I				
Ang II (10^{-8} M) + 6.90 μM A-IGF-I	1888.385**	207.5	1940.302**	211.0
Ang II (10^{-8} M) + 13.80 μM A-IGF-I	1876.937**	206.2	1928.371**	209.8
1.38 μM A-IGF-I				
6.90 μM A-IGF-I				
13.80 μM A-IGF-I	1375.123**	151.1	1379.654**	150.1

Table 1. Antisense IGF-I. Statistical analysis was performed using ANOVA with Fisher PLSD. *95% significant according to Fisher PLSD using ANOVA. **99% significant according to Fisher using ANOVA.

Addition	Experiment II			
	Day 1		Day 2	
	Size (μM2)	Percent of Control	Size (μM2)	Percent of Control
None (Control)	907.890	100.0	910.485	100.0
Ang II (10^{-8} M)	1146.800**	126.3	1396.859**	153.4
Ang II (10^{-8} M) + 1.38 μM A-IGF-I				
Ang II (10^{-8} M) + 6.90 μM A-IGF-I	1898.295**	209.1	1939.948**	213.1
Ang II (10^{-8} M) + 13.80 μM A-IGF-I	1799.362**	198.2	1790.371**	196.6
1.38 μM A-IGF-I				
6.90 μM A-IGF-I				
13.80 μM A-IGF-I	1425.123**	157.0	1456.654**	160.0

Table2 . Antisense IGF-I. Statistical analysis was performed using ANOVA with Fisher PLSD. *95% significant according to Fisher PLSD using ANOVA. **99% significant according to Fisher using ANOVA.

Addition	Experiment III			
	Day 1		Day 2	
	Size (μM2)	Percent of Control	Size (μM2)	Percent of Control
None (Control)	903.761	100.0	901.644	100.0
Ang II (10^{-8} M)	1137.465**	125.9	1330.168**	147.5
Ang II (10^{-8} M) + 1.38 μM A-IGF-I	1904.632**	210.7	1907.311**	211.5
Ang II (10^{-8} M) + 6.90 μM A-IGF-I	1880.374**	208.1	1872.189**	207.6
Ang II (10^{-8} M) + 13.80 μM A-IGF-I	1803.813**	199.6	1788.437**	198.4
1.38 μM A-IGF-I	1140.376*	126.2	1144.331**	126.9
6.90 μM A-IGF-I	1298.388**	143.6	1302.118**	144.4
13.80 μM A-IGF-I	1394.372**	154.3	1399.225**	155.2

Table 3. Antisense IGF-I. Statistical analysis was performed using ANOVA with Fisher PLSD. *95% significant according to Fisher PLSD using ANOVA. **99% significant according to Fisher using ANOVA.

Addition	Experiment I			
	Day 1		Day 2	
	Size (μM2)	Percent of Control	Size (μM2)	Percent of Control
None (Control)	910.153	100.0	919.361	100.0
Ang II (10^{-8} M)	1145.750**	125.9	1434.309**	156.0
Ang II (10^{-8} M) + 0.750 μM A-IGF-II				
Ang II (10^{-8} M) + 1.885 μM A-IGF-II				
Ang II (10^{-8} M) + 5.000 μM A-IGF-II				
Ang II (10^{-8} M) + 9.425 μM A-IGF-II	934.631*	102.7	950.671*	103.4
Ang II (10^{-8} M) + 18.850 μM A-IGF-II	920.461	101.1	925.956	100.7
0.750 μM A-IGF-II				
1.885 μM A-IGF-II				
5.000 μM A-IGF-II				
9.425 μM A-IGF-II				
18.850 μM A-IGF-II	910.439	100.0	923.859	100.5

Table 4. Antisense IGF-II. Statistical analysis was performed using ANOVA with Fisher PLSD. *95% significant according to Fisher PLSD using ANOVA. **99% significant according to Fisher using ANOVA.

Addition	Experiment II			
	Day 1		Day 2	
	Size (μM2)	Percent of Control	Size (μM2)	Percent of Control
None (Control)	907.890	100.0	910.485	100.0
Ang II (10^{-8} M)	1146.800**	126.3	1396.859**	153.4
Ang II (10^{-8} M) + 0.750 μM A-IGF-II				
Ang II (10^{-8} M) + 1.885 μM A-IGF-II				
Ang II (10^{-8} M) + 5.000 μM A-IGF-II				
Ang II (10^{-8} M) + 9.425 μM A-IGF-II	923.583*	101.7	933.671*	102.5
Ang II (10^{-8} M) + 18.850 μM A-IGF-II	915.737	100.9	926.432	101.8
0.750 μM A-IGF-II				
1.885 μM A-IGF-II				
5.000 μM A-IGF-II				
9.425 μM A-IGF-II				
18.850 μM A-IGF-II	917.097	101.0	924.227	101.5

Table 5. Antisense IGF-II. Statistical analysis was performed using ANOVA with Fisher PLSD. *95% significant according to Fisher PLSD using ANOVA. **99% significant according to Fisher using ANOVA.

Addition	Experiment III			
	Day 1		Day 2	
	Size (μM2)	Percent of Control	Size (μM2)	Percent of Control
None (Control)	904.497	100.0	905.471	100.0
Ang II (10^{-8} M)	1177.949**	130.2	1396.863**	154.3
Ang II (10^{-8} M) + 0.750 μM A-IGF-II	1037.507**	114.7	1039.471**	114.8
Ang II (10^{-8} M) + 1.885 μM A-IGF-II	955.199**	105.6	955.989*	105.6
Ang II (10^{-8} M) + 5.000 μM A-IGF-II	948.392**	104.9	950.461*	105.0
Ang II (10^{-8} M) + 9.425 μM A-IGF-II	929.005*	102.7	931.419*	102.9
Ang II (10^{-8} M) + 18.850 μM A-IGF-II				
0.750 μM A-IGF-II	910.440	100.7	911.439	100.7
1.885 μM A-IGF-II	909.461	100.5	912.976	100.8
5.000 μM A-IGF-II	908.000	100.4	910.778	100.6
9.425 μM A-IGF-II	909.432	100.5	909.761	100.5
18.850 μM A-IGF-II				

Table 6. Antisense IGF-II. Statistical analysis was performed using ANOVA with Fisher PLSD. *95% significant according to Fisher PLSD using ANOVA. **99% significant according to Fisher using ANOVA.

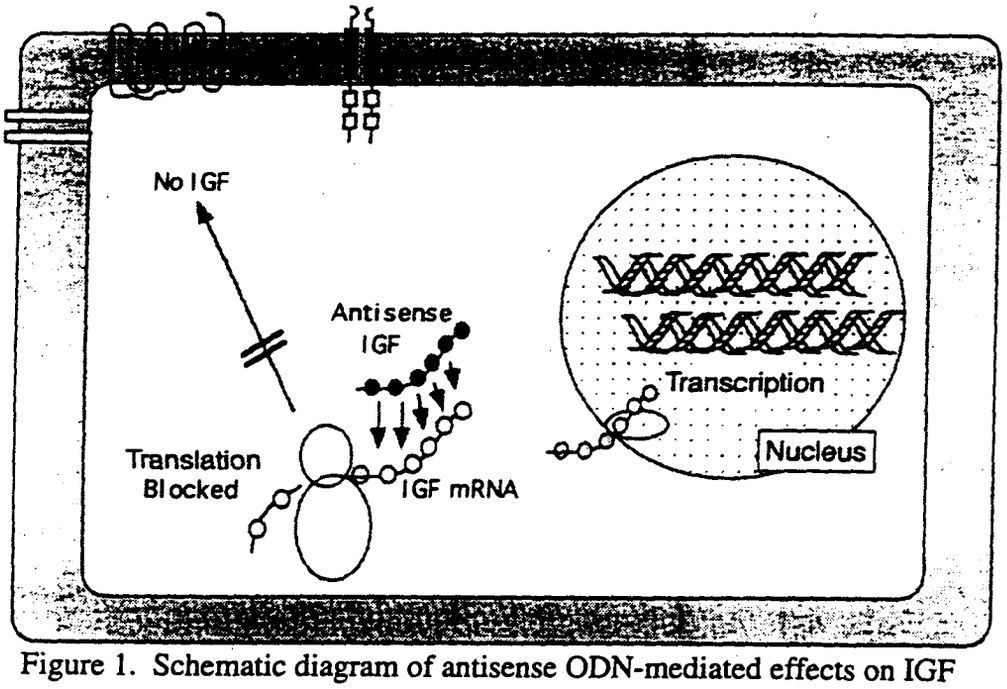

Figure 1. Schematic diagram of antisense ODN-mediated effects on IGF synthesis.

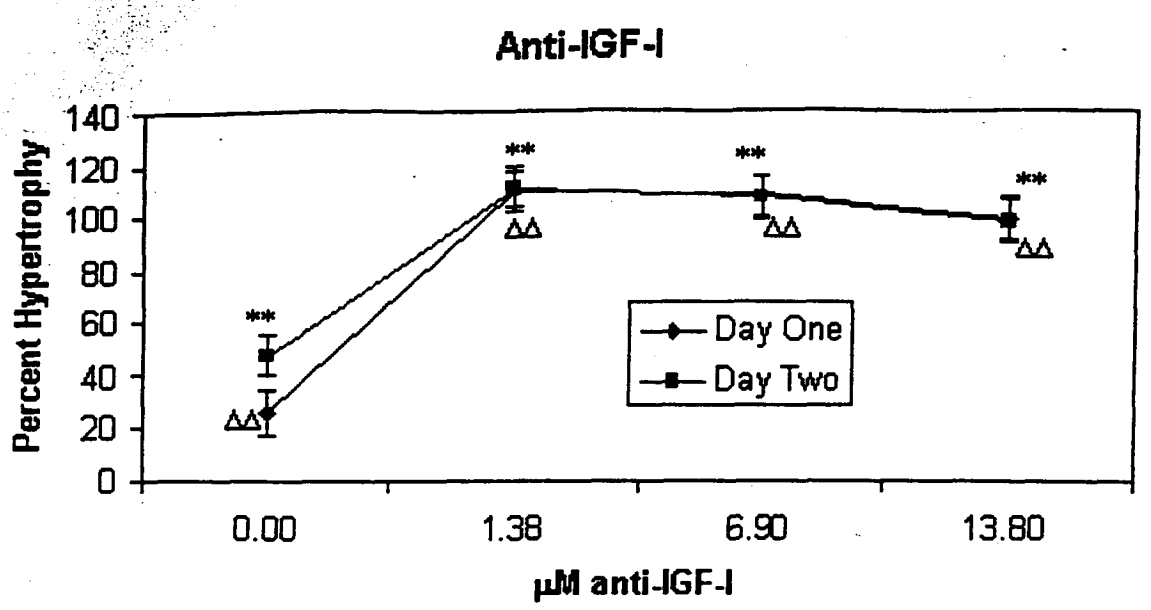

Figure 2. Dose-response of antisense IGF-I on cell hypertrophy in adult rat cardiomyocytes. Cells were treated with various concentrations of IGF-I at day 0 and day 1. Deviations are reported as mean +/-SD. ΔΔ signifies 99% significance for day one and ** denotes 99% statistical significance for day two.

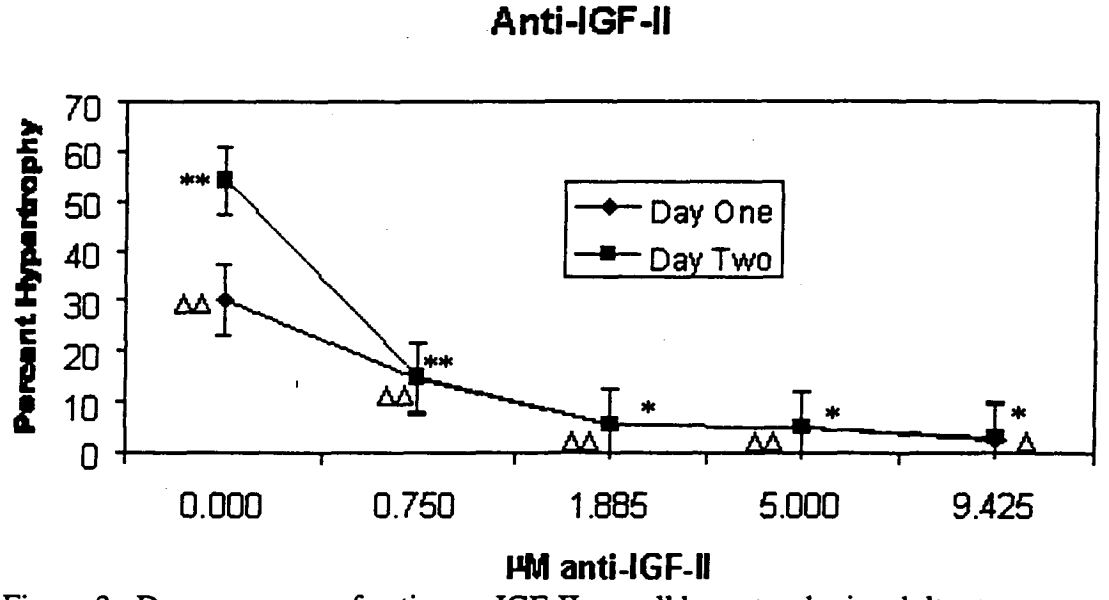

Figure 3. Dose-response of antisense IGF-II on cell hypertrophy in adult rat cardiomyocytes. Cells were treated with various concentrations of IGF-II at day 0 and day 1. Deviations are reported as mean +/-SD. ΔΔ signifies 99% significance(95% for Δ) for day one and ** denotes 99% (95% significance signified by *) statistical significance for day two.