Effect of inhibitors of signal transduction on IGF-1-induced protein synthesis associated with hypertrophy in cultured neonatal rat ventricular myocytes

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Abstract IGF-1 increased 2-fold protein synthesis in cardiac myocytes. Genistein, whether added during preincubation or with IGF-1 at the start of incubation, significantly inhibited the IGF-1-induced stimulation of protein synthesis, autophosphorylation of the β-subunit of IGF-1 receptor and inhibition of ERK. When added 1 or 6 h after IGF-1, however, genistein was without effect. IGF-1-stimulated protein synthesis was also significantly inhibited by PD-098059, staurosporine, and rapamycin, but not by wortmannin, in cardiac myocytes. Some inhibitors produced a reduction in cell size. Activation of the ERK cascade by IGF-1 may be responsible for some of the features associated with cardiac myocyte hypertrophy.

Key words: Insulin-like growth factor-1; Heart; Cardiac myocyte; Protein kinase; Signal transduction; Cardiac hypertrophy

1. Introduction

Left ventricular hypertrophy (LVH) is associated with important adverse cardiovascular events [1,2]. Although major advances have been made in the understanding of the general processes of growth and differentiation of myocardial cells, the biochemical basis of pathological cardiac hypertrophy is still poorly understood [3]. In response to hormonal and mechanical stimuli [4–9], the heart adapts by hypertrophy of individual ventricular myocytes. Because these cells are terminally differentiated and have lost the ability to proliferate, cardiac growth during hypertrophy results primarily from an increase in cellular protein content, with little or no change in the number of the cells themselves [10].

Insulin-like growth factor-1 (IGF-1) plays an essential role in the regulation of growth and development [11]. IGF-1 and its receptor occur in the neonatal rat myocardium, where IGF-1 may act in an autocrine or paracrine manner [12]. There is also clinical and experimental evidence for a role of IGF-1 in the initiation and development of LVH [13–20]. Because IGF-1 is cardioprotective [17,21–23], an understanding of its action at the molecular level is of practical concern.

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Abbreviations: ERK, extracellular signal-regulated kinase; GN, genistein; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; IRS-1, insulin receptor substrate-1; MBP, myelin basic protein; MEK, ERK activating kinase; PD, PD-098059; PI3-K, phosphatidylinositol 3-kinase; p90 RSK, p90 S6 kinase; RP, rapamycin; ST, staurosporine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; WM, wortmannin

IGF-1 activates multiple and complex signal transduction pathways, some of which may be relevant to the hypertrophic response of the heart [24]. Activation of IGF-1R in these cells is followed by autophosphorylation of its two β-subunits, by increases in the phosphotyrosine content of ERKs, IRS-1, phospholipase C-γ1, and PI3-K. IGF-1 also activates specific PKC isozymes, the ERK cascade and p90 RSK sequentially, as well as stimulating protein, but not DNA, synthesis ([24], and unpublished observations).

The aim of this work was the further elucidation of the role of the IGF-1 receptor in the development of hypertrophy in neonatal rat ventricular myocytes by modulation of its signal transduction system.

2. Materials and methods

2.1. Chemical and biochemical reagents

[γ-^{32}P]ATP and [^{3}H]phenylalanine were from NEN-Dupont (Boston, MA, USA). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), Dulbecco's modified Eagle's medium (DMEM), medium 199 (M199), protease inhibitors, bovine myelin basic protein (MBP), staurosporine, ATP, genistein, wortmannin, rapamycin and other biochemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Heat-inactivated fetal calf serum (FCS), horse serum and other tissue culture products were from Life Technologies Inc. (Gaithersburg, MD, USA). Antibodies raised against tyrosine-phosphorylated proteins (anti-phosphotyrosine antibody clone 4G10) and ERK were purchased from UBI (Lake Placid, NY, USA). ECL immunoblotting detection reagents, autoradiographic film and prestained molecular mass standard proteins were from Amersham International. Protein assay reagents were from Bio-Rad (Richmond, VA, USA). Human recombinant IGF-1 was donated by Drs. P. Valenzuela and C. George-Nascimento (Chiron Corp., CA, USA). PD-098059 (PD) was a gift from Dr. A.M. Saltiel (Parke-Davis, MI, USA).

2.2. Culture and treatment of ventricular myocytes

Neonatal ventricular myocytes were prepared from hearts of 1–3-day-old Sprague-Dawley rats (Animal Breeding Facility, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile) as described previously [24]. The myocytes, plated at a final density of 1.4×10^{3}/mm^{2} on gelatin-precoated 60 mm dishes, were confluent and spontaneously beating after 18 h. Serum was withdrawn for 24 h before the cells were further treated with agonists (IGF-1 10 nM or TPA 1 μM) in serum-free medium (DMEM-M199) at 37°C.

2.3. Protein synthesis

Confluent cells were grown to quiescence in serum-free medium for 24 h. Cardiac myocytes were preincubated for 30 min in fresh serum-free medium in the presence or absence of genistein (a protein tyrosine kinase inhibitor, GN, 100 μM), PD-98059 (a MEK inhibitor, PD, 100 μM), wortmannin (a PI3-K inhibitor, 100 nM), staurosporine (a protein tyrosine kinase and protein kinase C inhibitor, ST, 1 μM) and rapamycin (a p90-rsk inhibitor, RP, 20 nM). Cells were then stimulated with IGF-1 (10 nM) in fresh serum-free medium for 24 h in the presence of [^{3}H]phenylalanine (5 μCi/well) and the inhibitor. The cells were washed with ice-cold PBS and 10% (w/v) trichloroacetic acid was

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added at 4°C for 60 min to precipitate the proteins. The precipitates were washed twice with ethanol and dissolved in 1 M NaOH; the resulting solutions were neutralized with 1 M HCl and counted in a liquid scintillation counter.

2.4. IGF-1R tyrosine phosphorylation

Tyrosine phosphorylation of β-subunits of IGF-1R was analyzed by immunoblotting with an anti-phosphotyrosine antibody as previously described [24]. Briefly, confluent cardiac myocytes in 60 mm dishes were exposed to IGF-1 (10 nM) for 1 min, in the presence or absence of GN (10–100 μM) in serum-free medium at 37°C. Medium was then removed by aspiration and the cells were washed twice with cold Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline. Myocytes were scraped into 150 μl of cold lysis buffer (see [24]). The protein content of the lysate was determined by Bradford's method [25] and equal amounts of protein were separated by SDS-PAGE on 9% gels, and then transferred electrophoretically to nitrocellulose membranes (0.45 μm). Tyrosine-phosphorylated β-subunits of IGF-1R were probed with a monoclonal anti-phosphotyrosine antibody and detected with horseradish peroxidase-conjugated anti-mouse immunoglobulin using an ECL system [24].

2.5. In-gel ERK assay

Activities of MBP kinases were determined with an in-gel kinase assay [26]. Briefly, cells were treated with agonists, lysates were prepared, and proteins resolved in 10% SDS-polyacrylamide gels containing 0.5 mg/ml MBP. ERK1 and ERK2 activities were detected by incubation of these gels with [γ-^{32}P]ATP and autoradiography as described earlier [26].

2.6. Expression of results and statistical methods

Results are expressed either as means ± S.E.M. for the number of independent experiments indicated (*n*) or as examples of representative experiments performed on at least two separate occasions. Statistical analysis was performed using ANOVA, and comparisons between groups were performed using a protected Tukey's *t*-test.

3. Results

3.1. Time-course of the effect of IGF-1 on protein synthesis in rat ventricular myocytes

After 1, 6 or 24 h exposure of confluent cardiac myocytes to 10 nM IGF-1, protein synthesis was increased 1.9-, 1.7-, and 2.2-fold over control levels, respectively (Fig. 1) in the absence of any stimulation of [^{3}H]thymidine incorporation (evaluated at 24 h). Cell size was also increased after 24–48 h, although to a lesser extent than observed with phenylephrine (50 μM).

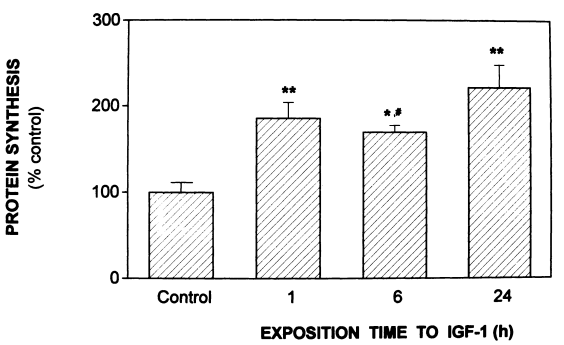

Fig. 1. Effect of IGF-1 on the stimulation of protein synthesis in cultured neonatal cardiac myocytes. Cells were incubated for 24 h in serum-free medium containing L-[2,6-^{3}H]phenylalanine with or without IGF-1 (10 nM). Rates of protein synthesis were determined as described in Section 2. Results are means ± S.E.M. of four independent experiments. Control = 31 504 ± 7486 dpm; $*P < 0.01$ and $**P < 0.01$ vs. control; $P < 0.05$ vs. IGF-1 (24 h).

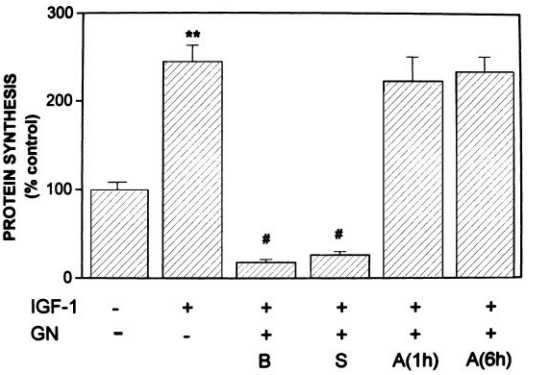

Fig. 2. Differential time effect of genistein on IGF-1-induced stimulation of protein synthesis in cultured cardiac myocytes. Cells were incubated for 24 h in serum-free medium containing L-[2,6-^{3}H]phenylalanine and IGF-1 (10 nM) with or without genistein (GN, 100 μM), which was added 1 h before (B), simultaneously (S), or at 1 or 6 h (A1h, A6h, respectively) after incubation with IGF-1. Rates of protein synthesis were determined as described in Section 2. Results are means ± S.E.M. of three independent experiments. Control = 12 820 ± 6444 dpm, $**P < 0.01$ vs. control; $P < 0.01$ vs. cells incubated with IGF-1 alone.

3.2. Time differential effect of genistein on IGF-1-induced protein synthesis in cardiac cells

Fig. 2 shows that genistein, whether added during preincubation or with IGF-1 at the start of incubation, significantly inhibited, to values lower than those of controls, the IGF-1-induced stimulation of protein synthesis. When added 1 or 6 h after IGF-1, however, genistein was without effect.

3.3. Effect of genistein on IGF-1R autophosphorylation and ERK activation in cultured ventricular myocytes

As shown in Fig. 3A, incubation of cardiac myocytes with IGF-1 resulted in increases in the phosphotyrosine content of two proteins with apparent molecular masses of 96 and 100 kDa, corresponding to the two β-subunits of IGF-1R. IGF-1R autophosphorylation (particularly of the 96 kDa band) was much lower in neonatal cardiac myocytes preincubated with genistein (10–100 μM) added before IGF-1. Low levels of receptor autophosphorylation were observed when genistein was added alone. After membranes were stripped and reblotted with a polyclonal antibody directed against the β-subunit of IGF-1R, we confirm both the presence of two isoforms of the β-subunit of IGF-1R and that similar amounts of IGF-1R protein were present in all samples (data not shown). There were significant changes in the intensity of the ERK1 band in the in-gel ERK assay after the pre-exposure of myocytes to genistein and subsequent addition of IGF-1 (Fig. 3B).

3.4. Effect of different chemical inhibitors on IGF-1R signaling in cultured ventricular myocytes

IGF-1-stimulated phenylalanine incorporation was significantly inhibited by GN, PD, ST and RP, but not WM, in cardiac myocytes (Fig. 4). GN, PD and bisindolylmaleimide I (a specific PKC inhibitor) produced an appreciable reduction in cell size (data not shown). Cell viability (approximately 85%) was unaffected by exposure to the above inhibitors.

4. Discussion

Cardiac hypertrophy is a compensatory adaptation of car-

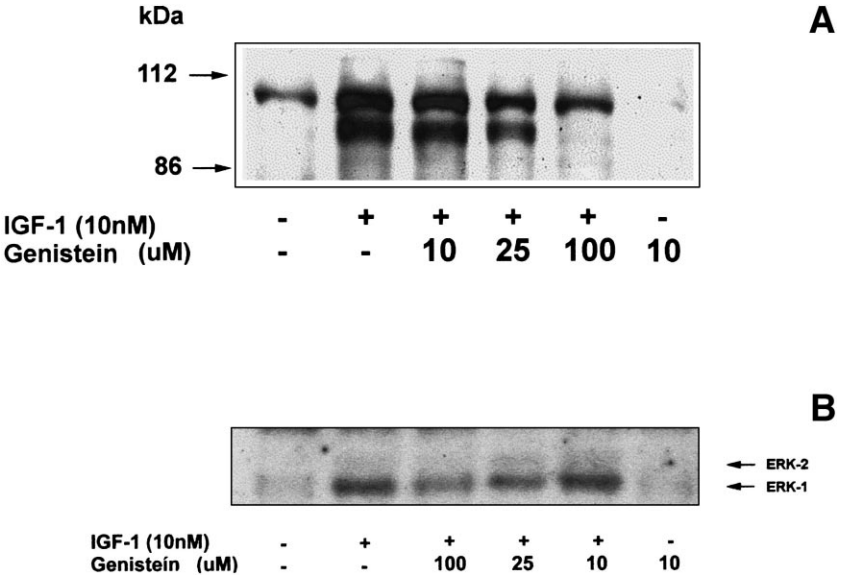

Fig. 3. Effect of genistein on the IGF-1-induced stimulation of phosphorylation of the β-subunit of IGF-1R and ERK activity in cultured neonatal cardiac myocytes. Lysates of cells pretreated for 30 min with increasing concentrations of genistein (GN) before exposure for 1 min to IGF-1 (10 nM) were subjected to SDS-PAGE. The β-subunits of IGF-1R containing phosphotyrosine were detected by Western immunoblotting as described in Section 2 (A). Denaturated samples were also subjected to SDS-PAGE in 10% gels containing 0.2 mg/ml MBP. B: A representative result of the phosphorylation of MBP assayed in situ as described in Section 2. The arrows to the right of this panel indicate the position of ERK2 and ERK1. Results are representative of three independent experiments. The positions of prestained molecular weight markers (kDa) are indicated to the left of A.

diac myocytes to increased hemodynamic stress or to loss of contractile myocytes. In addition to an overall increase in cell size, protein and RNA content, there are characteristic alterations at the level of gene expression [7]. One of the most attractive hypotheses is that trophic factors (such as IGF-1, angiotensin II, endothelin, etc.) are produced by cardiac nonmuscle cells or by the myocytes themselves in response to mechanical stress, and that these factors, through intracellular signaling cascades coupled to specific cell surface receptors, regulate protein synthesis and transcription of genes of the contractile apparatus, as well as other genes involved in cell growth. We have demonstrated that short exposure (1 h or less) of ventricular myocytes to IGF-1 leads to the development of hypertrophy and that this requires a fully functional IGF-1 receptor. Our results also show that the PKC-ERK pathway, but not PI3-K, could be involved downstream of IGF-1R.

Some studies have involved PKC in the development of hypertrophy (reviewed in [10]). Both direct activation of PKC by transfection with plasmids encoding constitutively active PKC [27,28] or exposure to TPA [28–30] leads to development of hypertrophy in cardiac myocytes. It has also been suggested that a consequence of the activation of PKC is the stimulation of the ERK cascade and this effect may also be relevant to the hypertrophic response [31,32]. The ERK cascade can be activated by several hypertrophic stimuli (fibroblast growth factors, ET-1 and α_1-adrenergic agonists, TPA) and by stretching [31–33], and, to a lesser extent, by bradykinin [34]. This has prompted the suggestion that the activation of ERK could be a convergence point through which different stimuli exert a common hypertrophic action. We have observed that ERK activation by IGF-1 is linked to PKC in neonatal cardiac myocytes (unpublished data). It is unresolved whether the ERK pathway is obligatory for the growth response of cardiac myocytes to hypertrophic agonists [31,35–40]. Our results do, however, show that inhibition of the ERK cascade (at the level of MEK and p90-rsk) leads to a significant decrease in IGF-1-stimulated protein synthesis. Activation of the ERK family may be essential for the activation of gene expression in response to hypertrophic agonists rather than for the changes in morphology [41]. Our data imply that activation of the ERK cascade by IGF-1 may be responsible, at least in part, for the morphological changes and increased protein synthesis associated with the development of ventricular myocyte hypertrophy.

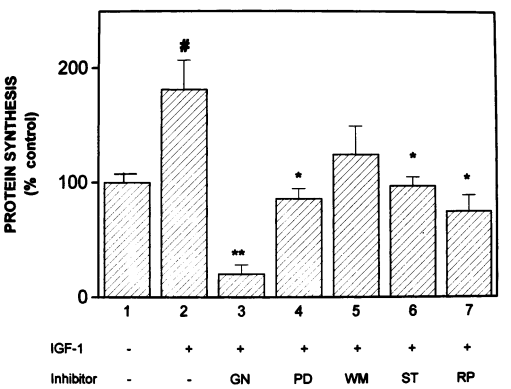

Fig. 4. Effect of different inhibitors on the stimulation of protein synthesis in cultured cardiac myocytes. Cells were preincubated in serum-free medium with or without GN (100 μM), PD (100 μM), WM (100 nM), ST (1 μM) or RP (20 nM) for 30 min at 37°C. The medium was then replaced with medium containing L-[2,6-^{3}H]phenylalanine and IGF-1 (10 nM) with or without inhibitor. After 24 h exposure, rates of protein synthesis were determined as described in Section 2. Results are means ± S.E.M. of three independent experiments. Control = 14 194 ± 2464; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. IGF-1 alone; $P < 0.01$ vs. control.

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