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Angiotensin-converting enzyme (ACE) inhibition attenuates insulin-like growth factor-I (IGF-I) induced cardiac fibroblast proliferation

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1 The effects of angiotensin-converting enzyme (ACE) inhibition and angiotensin type 1 (AT_1) receptor blockade on insulin-like growth factor-I (IGF-I) induced proliferation and immediate-early-gene expression of neonatal rat cardiac fibroblasts were investigated. Moreover the role of the IGF-I receptor (IGF-IR) in this process was evaluated.

2 IGF-I (10^{-9}–10^{-7} M) stimulated neonatal rat cardiac fibroblast growth in a dose-dependent fashion (maximum: 3.5 ± 0.1 fold, 10^{-7} M), as determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation. ACE inhibition or AT_1 receptor blockade attenuated the IGF-I (10^{-7} M) induced neonatal rat cardiac fibroblast growth in a concentration-dependent fashion (moexiprilat: $50\pm2\%$, enalaprilat: $31\pm2\%$, CV11974; $58\pm1\%$, all: 10^{-7} M).

3 IGF-I stimulated cellular growth was accompanied by an upregulation of the immediate early genes c-Fos (2.4 ± 0.3 fold), Egr-1 (4.7 ± 1.1 fold) and Sp1 (6.2 ± 0.7 fold). IGF-I induced expression was completely inhibited by ACE inhibition or AT_1 receptor blockade.

4 Stimulation with IGF-I or Ang II (10^{-7} M) increased IGF-IR expression 5.7 ± 0.5 fold and 3.6 ± 0.5 fold respectively. The IGF-I induced overexpression of the IGF-IR was reduced by ACE inhibition with moexiprilat (10^{-7} M) by $79\pm7\%$ and by AT_1 receptor blockade with CV11974 (10^{-7} M) by $79\pm5\%$.

5 These data demonstrate that the mitogenic action of IGF-I in neonatal rat cardiac fibroblasts is in part mediated by activation of the renin-angiotensin system (RAS) with subsequent upregulation of IGF-IR expression. This observation has important implications for the treatment of cardiac diseases with ACE inhibitors alone and their combination with IGF-I or growth hormone.
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Abbreviations: ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT_1 receptor, angiotensin type 1 receptor; BrdU, 5-bromo-2′deoxyuridine; DMEM, Dulbecco's modified Eagle medium; FCS, foetal calf serum, GH, growth hormone; HS, horse serum; IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; PAGE, Polyacrylamide gel electrophoresis; RAS, renin-angiotensin system; SDS, sodium dodecyl sulphate

Introduction

The renin-angiotensin system (RAS) as well as growth hormone (GH) and its main mediator of action, insulin-like growth factor-I (IGF-I), play important roles in the pathogenesis of cardiac diseases. Blockade of the RAS *via* angiotensin-converting enzyme (ACE) inhibition is well established in the treatment of heart failure (Brown & Vaughan, 1998). An excess of GH (endocrine action in patients with acromegaly) or IGF-I (autocrine/paracrine action *via* overexpression in the cardiac cells) is involved in the pathogenesis of left ventricular hypertrophy, hypertrophic cardiomyopathy and interstitial fibrosis (Frustaci *et al*., 1999; Li *et al*., 1997a; Lombardi *et al*., 1997). Furthermore, recent studies have shown that substitution with GH or IGF-I can exert beneficial effects on cardiac function, contractile reserve, apoptotic signalling and myocyte proliferation in heart failure (Clark, 1997; Li *et al*., 1997b; Ross, 1999; Tajima *et al*., 1999). All clinical trials to test the effectiveness of IGF-I or GH as an adjunctive therapy in patients with heart failure, were based on the cotreatment with ACE inhibitors (Brown & Vaughan, 1998). Several studies have shown that Ang II modulates the expression of multiple components of the IGF-I system in vascular smooth muscle cells (Delafontaine, 1998; Du *et al*., 1999; Gustafsson *et al*., 1999; Scheidegger *et al*., 1995). Furthermore, recent observations suggest that the interaction between cardiac and systemic IGF-I plays an important role in the cardiac remodelling response to Ang II (Brink *et al*., 1999). However, little is known about the interaction of IGF-I and ACE inhibition in cardiac disease. We therefore studied the effects of ACE inhibition on IGF-I induced proliferation and changes in gene expression of neonatal rat cardiac fibroblasts.

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Methods

Cell isolation

Isolation of cardiac fibroblasts from neonatal rats was performed according to a modified protocol as described previously (Grohé *et al*., 1997). Briefly, the hearts of 1–2

day-old rats (Wistar-Kyoto) were isolated and digested with 10 ml of Spinner-solution (in mM): NaCl 116, KCl 5.3, NaH_2PO_4 8, $NaHCO_3$ 22.6, HEPES 10, D-Glucose 5, pH 7.4, containing 0.1% collagenase (Cytogen, Berlin, Germany) for 10 min at 37°C in eight consecutive steps. After each digestion, the medium containing the suspended cells was removed and an equal volume of Spinner/collagenase solution was added. The cardiac cell suspension was mixed with an equal volume of Ham's F10 (Gibco BRL, Eggenstein, Germany) supplemented with 10% horse serum (HS; Biochrom, Berlin, Germany), 10% foetal calf serum (FCS; c.c.pro, Hamburg, Germany) and 25 μg ml^{-1} Gentamycin (Gibco BRL, Eggenstein, Germany) and stored at 4°C. Cells were centrifuged at $400 \times g$ for 5 min and the cell pellets were resuspended in 20 ml of Ham's F10 supplemented with 10% HS and 10% FCS and plated on culture dishes. After 75 min the medium, which contained the cardiomyocyte fraction of the digested tissue, was removed. The dishes were gently rinsed three times to remove remaining cardiomyocytes. The culture medium for cardiac fibroblasts was changed for Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Eggenstein, Germany) supplemented with 20% FCS and 25 μg/ml^{-1} Gentamycin. Purity of cardiac fibroblast culture (>97%) was assessed by repeated differential plating, microscopic evaluation and immunostaining (van Eickels *et al*., 1999; Reiss *et al*., 1995).

Proliferation assay

After 24 h incubation in serum-free medium (DMEM), cells (25,000 cells per well of a 96-microtiter plate), passages 2–3, were stimulated with IGF-I (10^{-9}–10^{-7} M) and coincubated with either the IGF-I analogon H-1356, that acts as an IGF-IR antagonist (Bachem Biochemica GmbH, Heidelberg, Germany; 10^{-4} M; Pietrzkowski *et al*., 1992), one of the following ACE inhibitors: moexiprilat (the bioactive metabolite of moexipril; Schwarz Pharma AG, Monheim, Germany; 10^{-9}–10^{-5} M; White *et al*., 1994), enalaprilat (the bioactive metabolite of enalapril; 10^{-9}–10^{-5} M) or the AT_1 receptor antagonist CV11974 (the bioactive metabolite of candesartan, Takeda Chemical Industries Ltd., Osaka, Japan; 10^{-7} M; Ojima *et al*., 1997). Cellular proliferation was assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation during the last 4 h of the 24 h incubation period using a colorimetric immunoassay according to the manufacturer's guidelines (Boehringer Mannheim, Mannheim, Germany) as described before (van Eickels *et al*., 1999). The extinctions were measured at 450 nm in an ELISA plate reader (Titertek multiscan plus, EFlab, Helsinki, Finland). All values consist of an $n=9$.

Immunoblot analysis

Neonatal rat cardiac fibroblasts, passages 2–3, were starved for 24 h in serum-free medium. After exposure to IGF-I, moexiprilat or CV11974 (10^{-7} M) the cells were lysed in 0.5 ml of the following buffer (mM): NaCl 50, Tris 20, (pH 7.4), NaF 50, EDTA 50, sodium pyrophosphate ($Na_4P_2O_7$) 20, sodium orthovanadate (Na_3VO_4) 1, 1% triton X-100, PMSF 1, 0.6 mg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin. Protein content was measured with a standard Bradford assay. Total cell lysates (40 μg/lane) were analysed by sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE) and electrophoretically transferred to a nitrocellulose membrane. Immunoblotting was performed with polyclonal antibodies against Egr-1 (sc-110, 1:500), c-Fos (sc-52, 1:500), Sp1 (sc-59, 1:500) and the β subunit of the IGF-IR (sc-713, 1:250) as described previously (all antibodies: Santa Cruz Biotechnology Inc., Heidelberg, Germany (van Eickels *et al*., 1999). A horseradish peroxidase-labelled goat anti-rabbit IgG antibody (Amersham, Buckinghamshire, U.K., 1:5000) was used as secondary antibody, followed by detection with the enhanced chemiluminescence technique (ECL, Amersham, Buckinghamshire, U.K.). Densitometric analysis was performed on an Epson GT 8000 scanner using the analysis software ScanPack (Biometra, Göttingen, Germany).

Materials

All chemicals were obtained from Merck, Darmstadt, Germany and Sigma Chemicals, Deisenhofen, Germany if not otherwise specified.

Statistical evaluation

All reported values are expressed as mean ± s.e.mean of 3–9 observations. Statistical comparisons were performed with one-way analysis of variance followed by Scheffé's test. A level of $P<0.05$ was accepted as statistically significant.

Results

Fibroblast proliferation

IGF-I induced neonatal rat cardiac fibroblast growth in a dose-dependent fashion as measured by BrdU incorporation (Figure 1a). This effect (10^{-7} M) was completely inhibited by coincubation with the IGF-IR antagonist H-1356 (10^{-7} M). Coincubation with the ACE inhibitors moexiprilat or enalaprilat or the AT_1 receptor antagonist CV11974 (10^{-7} M) led to a 50±2, 31±2 and 58±1% reduction of the IGF-I (10^{-7} M) induced growth respectively (Figure 1a). The antiproliferative effect of the ACE inhibitors on IGF-I induced growth was concentration-dependent, with a maximum inhibition of 71±2%, 52±1% and an IC_{50} of 41±1 nM, 90±1 mM for moexiprilat and enalaprilat respectively (Figure 1b).

Immediate early gene expression

Immediate early genes such as Egr-1, c-Fos and Sp1 are critically involved in cardiac fibroblast proliferation (van Eickels *et al*., 1999; Grohé *et al*., 1998). Stimulation with IGF-1 (10^{-7} M) caused a significant increase in the expression of Egr-1, c-Fos and Sp1 after 60 min. This induction was completely inhibited by coincubation with the IGF-IR antagonist H-1356 (10^{-7} M), the ACE inhibitor moexiprilat (10^{-7} M) or the AT_1 receptor antagonist CV11974 (10^{-7} M) (Figure 2).

IGF-I receptor expression

Previous studies have shown that the proliferative response of cells directly correlates with the amount of the IGF-IR present (Rubin & Baserga, 1995). We therefore studied the expression of IGF-IR in IGF-I stimulated neonatal rat cardiac fibroblasts. Immunoblot analysis of neonatal rat cardiac fibroblasts cultured in serum-free medium for 24 h revealed a low basal IGF-IR expression. IGF-I (10^{-7} M) led to a 5.7±0.5 fold increase of IGF-IR expression. Coincuba-

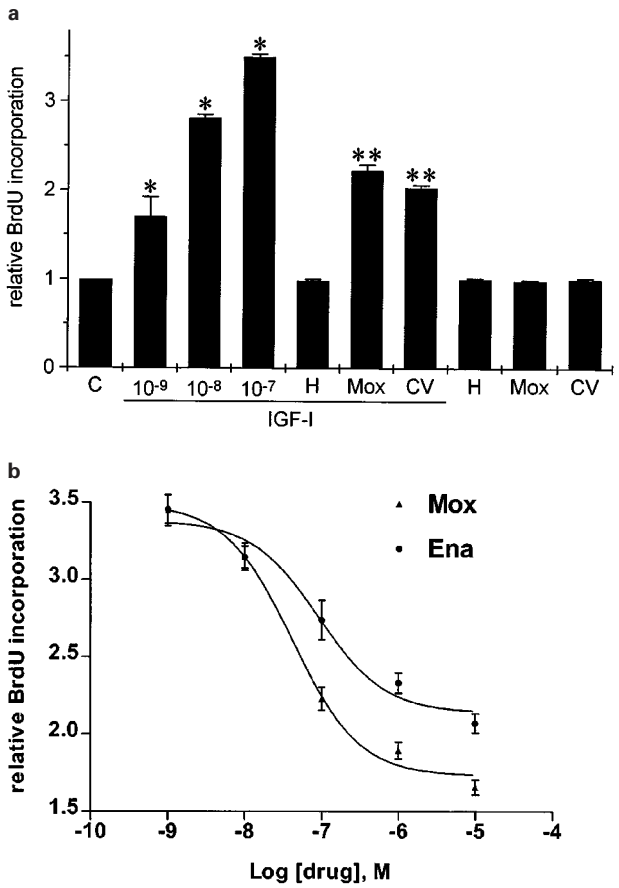

Figure 1 IGF-I induced cardiac fibroblast growth can be attenuated by ACE inhibition and by AT_1 receptor antagonism. (a) Starved neonatal rat cardiac fibroblast were stimulated with IGF-I at the indicated concentrations or coincubated with IGF-I (10^{-7} M) and the IGF-I receptor antagonist H-1356 (H; 10^{-7} M), moexiprilat (Mox; 10^{-7} M) or CV11974 CV; 10^{-7} M). Control cells were treated with vehicle (C) and with the inhibitors (H, Mox, CV) alone. Bars represents the relative BrdU incorporation compared to control cells (C). $*P<0.01$ vs control; $**P<0.01$ vs control and vs IGF-I (10^{-7} M) ($n=9$). (b) Dose-response curves for moexiprilat (Mox) and enalaprilat (Ena) in neonatal rat cardiac fibroblasts stimulated with IGF-I (10^{-7} M) ($n=9$).

tion with moexiprilat (10^{-7} M) or CV11974 (10^{-7} M) led to a 79 ± 7 and a $79\pm5\%$ reduction of the IGF-I induced IGF-IR expression (Figure 3). To further support the hypothesis that the activation of the RAS is involved in the growth response to IGF-I, we studied the effect of Ang II (10^{-7} M) on IGF-1R expression in neonatal rat cardiac fibroblasts. Ang II (10^{-7} M) led to a 3.8 ± 0.5 fold induction of IGF-IR expression. Coincubation with CV11974 (10^{-7} M) led to a complete inhibition of the Ang II induced IGF-IR expression (Figure 4). All immunoblot experiments were repeated with the ACE inhibitor enalaprilat showing similar results (data not shown).

Discussion

It has been shown previously that IGF-I and Ang II play important roles in cardiac fibroblast proliferation (van Eickels *et al*., 1999; Reiss *et al*., 1995). However, the interaction of these two mitogenic pathways is poorly understood. Here we show that IGF-I induces neonatal rat cardiac fibroblast proliferation and the expression of growth-related genes in neonatal rat cardiac fibroblasts. The observed effects of IGF-I were mediated *via* the activation of the IGF-IR, since they could be completely blocked by coincubation with the IGF-IR antagonist H-1356.

Interestingly, ACE inhibition also attenuated IGF-I induced neonatal rat cardiac fibroblast proliferation. The fact that an AT_1 receptor antagonist was equally effective suggests that this antiproliferative effect of ACE inhibition is mediated by Ang II. However, since the IC_{50s} measured in the proliferation assays were higher than the ones determined in *in vitro* cell free assays, we cannot exclude that the inhibition of enzymes besides ACE itself like chymases may also be important. Taken together, the data indicate that IGF-I activates the RAS in neonatal rat cardiac fibroblasts. It was reported recently that IGF-I can reduce the activation of the RAS in cardiac myocytes (Leri *et al*., 1999a,b). In these studies, however, the effect of the RAS on cardiac fibroblasts were not taken into account. It has been shown previously that IGF-I can activate the RAS and induce Ang II synthesis in perfused ovaries (Yoshimura *et al*., 1996).

We have demonstrated previously that Ang II leads to an activation of mitogenic signalling pathways and neonatal rat

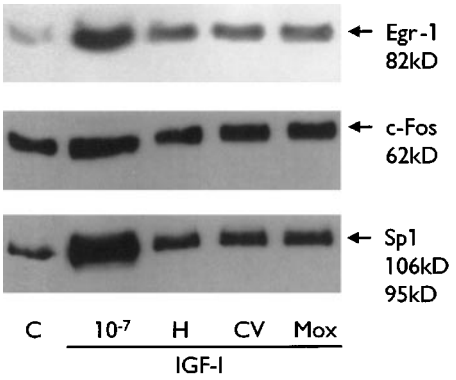

Figure 2 Immunoblot analysis revealed an increase of the immediate early genes Egr-1, c-Fos and Sp1 expression in starved neonatal rat cardiac fibroblasts stimulated with IGF-I (10^{-7} M, 60 min). This induction could be completely inhibited by a coincubation with the IGF-I receptor antagonist H-1356 (H; 10^{-7} M), moexiprilat (Mox; 10^{-7} M) or CV11974 (CV; 10^{-7} M). One of three similar experiments is shown.

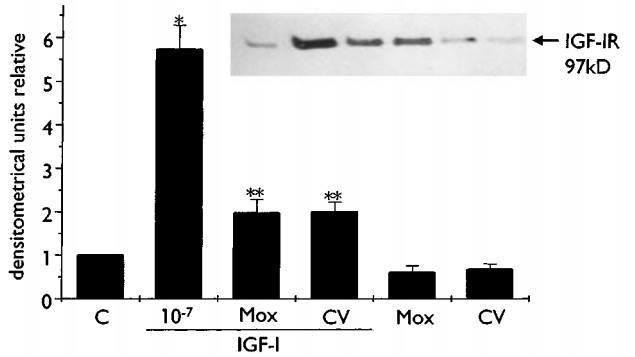

Figure 3 Immunoblot analysis of the IGF-IR expression in neonatal rat cardiac fibroblasts. Starved neonatal rat cardiac fibroblasts were stimulated for 24 h with IGF-I (10^{-7} M) and coincubated with moexiprilat (Mox; 10^{-7} M) and CV11974 (CV; 10^{-7} M). Control cells were treated with vehicle (C) and with the inhibitors (Mox, CV) alone. Densitometric analysis of three immunoblots. The values were normalized to cells treated with vehicle alone (C). $*P<0.01$.

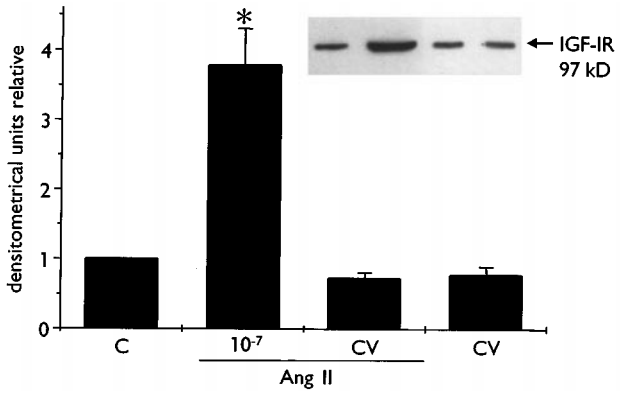

Figure 4 Immunoblot analysis of the IGF-IR expression in neonatal rat cardiac fibroblasts. Starved neonatal rat cardiac fibroblast were stimulated for 24 h with Ang II (10^{-7} M) and coincubated with CV11974 (CV; 10^{-7} M). Control cells were treated with vehicle (C) and with the inhibitor (CV alone). Densitometrical analysis of three immunoblots. The values were normalized to cells treated with vehicle alone (C). $^{*}P<0.01$.

cardiac fibroblast proliferation (van Eickels *et al*., 1999; Grohé *et al*., 1998). In addition to this direct mitogenic effect, we have shown in this study that the IGF-I induced activation of the RAS or the treatment with Ang II led to an increased expression of IGF-IRs in neonatal rat cardiac fibroblasts. Similar effects of Ang II on the expression of the components of the IGF-I system have been extensively studied in vascular smooth muscle cells (Anwar *et al*., 2000; Delafontaine & Lou, 1993; Du *et al*., 1999). Furthermore, a recent study in rats has shown that infusion of Ang II can lead to an increased expression of IGF-IR in the heart *in vivo*. The data observed in that study is difficult to compare with our results, since Ang II seems to modulate systemic factors like blood pressure and food intake *in vivo*, which may mask the direct actions of Ang II on the cardiac fibroblasts (Brink *et al*., 1999). Since the proliferative response of cells directly correlates with the amount of IGF-IR present (Rubin & Baserga, 1995), we hypothesize that the IGF-I mediated activation of the RAS leads to an increased expression of IGF-IRs and that this upregulation of IGF-IRs is crucial for the full mitogenic action of IGF-I in neonatal rat cardiac fibroblasts. The attenuation of IGF-I induced IGF-R expression could be one of the mechanisms by which ACE inhibition exerts its antiproliferative effects. However, other pathways activated by Ang II like the reduction of IGF-I binding protein 4 expression in vascular smooth muscle cells may also be important (Anwar *et al*., 2000).

Although our data were obtained in cardiac fibroblasts it is likely that similar effects can be observed in vascular smooth muscle cells. In a recent study by Gustafsson *et al*. (1999) a statistically not significant attenuation of thymidine incorporation into IGF-I stimulated vascular smooth muscle cells was observed after coincubation with the AT_1 antagonist losartan. Thus our findings may not only be important for cardiac fibrosis and remodelling, but may also play a role in vascular biology and vascular remodelling.

The observed interaction of Ang II and IGF-I offers new insights in the complex regulatory pathways, which lead to cardiac fibrosis. In addition, the results have important implication for future studies using GH or IGF-I for the treatment of heart failure. On the one hand ACE inhibition could be beneficial by attenuating fibroblast proliferation in patients treated with IGF-I or GH (Fazio *et al*., 1996). On the other hand ACE inhibition could interfere with the benefical effects of IGF-I or GH treatment on the cellular level (Osterziel *et al*., 1998). However, future *in vivo* studies and *in vitro* studies in cardiac fibroblasts and myocytes are needed to clarify the role of the interactions of ACE inhibition with IGF-I mediated signalling in the myocardium.

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