FEBS Letters 576 (2004) 156–160 FEBS 28834

Endothelin-1 is an essential co-factor for β_2-adrenergic receptor-induced proliferation of human cardiac fibroblasts

Neil A. Turner[a,*], David J. O'Regan[b], Stephen G. Ball[a], Karen E. Porter[a]

[a] *Institute for Cardiovascular Research, Worsley Building, University of Leeds, Leeds LS2 9JT, UK*
[b] *Department of Cardiac Surgery, The Yorkshire Heart Centre, Leeds General Infirmary, Leeds LS1 3EX, UK*

Received 6 July 2004; revised 4 August 2004; accepted 9 August 2004

Available online 18 September 2004

Edited by Veli-Pekka Lehto

Abstract **We previously demonstrated that chronic stimulation of the β_2-adrenergic receptor (β_2-AR) increases proliferation of cultured human cardiac fibroblasts (CF) via an autocrine mechanism. Here, we investigated the role of endothelin-1 (ET-1) in this process. ET_A-receptor antagonism or protein kinase C inhibition abolished the β_2-AR-induced increase in cell proliferation. RT-PCR and ELISA analysis demonstrated that although CF synthesized and secreted ET-1, this occurred independently of β_2-AR stimulation. Furthermore, despite activation of the MAP kinase pathway, ET-1 treatment did not stimulate CF proliferation. Therefore, the role of ET-1 in this process is that of an essential co-factor acting independently of β_2-AR stimulation.**

Keywords: Human; Cardiac fibroblast; β_2-Adrenergic receptor; Endothelin-1; Cell proliferation; Protein kinase C

1. Introduction

During the development of heart failure (HF), the left ventricle undergoes structural remodeling that serves initially as an important adaptive response, but is ultimately damaging [1]. An important contributor to the remodeling process in HF patients is an increase in myocardial sympathetic nervous activity [2]. Indeed, treatment of patients with β-adrenergic receptor (β-AR) antagonists (β-blockers) reduces adverse myocardial remodeling with an associated decrease in HF mortality [3]. A key component of the remodeling process is the transformation of quiescent cardiac fibroblasts (CF) into a more responsive myofibroblast phenotype that undergoes increased proliferation, invasion and extracellular matrix turnover [4]. In a previous study, we reported that chronic stimulation of the β_2-AR increased the rate of proliferation of human CF [5]. Hence, a potential mechanism by which β-blockers attenuate adverse myocardial remodeling in man is via inhibition of β_2-AR-induced CF proliferation. It is therefore an important aim to understand the mechanism by which activation of the β_2-AR leads to proliferation of human CF.

Endothelin-1 (ET-1) is a vasoactive peptide that contributes to the pathophysiology of HF [6]. Indeed, one of the most accurate markers for clinical outcome in HF patients is plasma concentrations of big endothelin, the immediate precursor of ET-1 [7]. Furthermore, animal studies have demonstrated that ET-1 plays a key role in catecholamine-induced myocardial remodeling [8,9]. ET-1 exerts its biological actions via two distinct cell surface receptors, ET_A-R and ET_B-R, which are coupled to protein kinase C (PKC) activation through stimulation of phospholipase C [10]. CF express both endothelin receptor (ET-R) subtypes [11–15] and their activation in rat CF can stimulate proliferation [16,17] and collagen turnover [18]. In addition to their ability to respond to ET-1, rat CF can also synthesize and secrete ET-1 in response to a variety of stimuli [13,19,20].

Given the ability of CF to both generate and respond to ET-1, and our prior observation that chronic β_2-AR stimulation enhances human CF proliferation via secretion of autocrine growth factors [5], we investigated whether ET-1 plays a role in β_2-AR-induced proliferation of human CF.

2. Materials and methods

2.1. Reagents

All cell culture reagents were purchased from Invitrogen (Paisley, UK), except fetal calf serum (FCS) that was from Biowest Ltd (Ringmer, East Sussex, UK). Isoproterenol (ISO) and human ET-1 were from Sigma (Poole, Dorset, UK). PD142893 and BQ123 were purchased from Alexis Biochemicals (Nottingham, UK) and BQ788 and GF1093203X were from Calbiochem (Nottingham, UK). All antibodies were from Cell Signaling Technology (Hitchin, Herts., UK).

2.2. Culture of human CF

Biopsies of right atrial appendage were obtained from patients without left ventricular dysfunction undergoing elective coronary artery bypass surgery. Local ethical committee approval and written informed patient consent were obtained. Primary cultures of CF were harvested, characterized as myofibroblasts, and cultured as described previously [5,21]. Experiments were performed on passage 2–4 cells from several patients.

2.3. Proliferation assays

Proliferation assays were performed essentially as described previously [5]. Briefly, CF were rendered quiescent with serum-free medium (SFM) for 48 h before exposure to minimal growth-promoting medium (MGM) containing 2.5% FCS and appropriate supplements.

* Corresponding author. Fax: +44-113-3434-803.
E-mail address: cvsnat@leeds.ac.uk (N.A. Turner).

Abbreviations: β_2-AR, β_2-Adrenergic receptor; CF, cardiac fibroblasts; CM, conditioned medium; ET-1, endothelin-1; ET-R, endothelin receptor; FCS, fetal calf serum; HF, heart failure; ISO, isoproterenol; MAPK, mitogen-activated protein kinase; MGM, minimal growth-promoting medium; PKC, protein kinase C; ppET-1, preproET-1; SFM, serum-free medium

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doi:10.1016/j.febslet.2004.08.080

Medium and drugs were replaced every 2 days and cell number determined after 7 days by counting viable cells using Trypan Blue and a hemocytometer. Using parallel cultures of CF, we generated control conditioned medium (C-CM) and ISO-CM as described previously [5]. Both C-CM and ISO-CM were supplemented with 1 μM alprenolol and 2.5% FCS before use.

2.4. Measurement of ppET-1 mRNA levels

CF were serum-starved for 48 h, then treated with fresh MGM for 24 h before addition of 1 μM ISO for 1–6 h. RNA was extracted and RT-PCR performed as described previously [5]. PreproET-1 (ppET-1) forward (5′-CAGCGCGGTGGGTGAGAACG-3′) and reverse (5′-CAAATGATGTCCAGGTGGCAGAAGTAG-3′) primers and GAPDH primers [5] were synthesized by Invitrogen. ppET-1 (135 bp) and GAPDH (240 bp) PCR products were resolved by 2% agarose gel electrophoresis and their relative intensity determined using a Typhoon 9410 Imager and ImageQuant software (Amersham Life Science). Reactions were optimized for the linear phase of PCR by serial dilution of RNA to ensure that band intensity was proportional to the amount of RNA template. Samples were then standardized for equal expression of GAPDH.

2.5. Measurement of ET-1 secretion

CF were cultured under identical conditions to those in the proliferation assay (Section 2.3). Cell culture supernatants were collected 24 and 48 h after addition of MGM with or without ISO. ET-1 quantification was performed using an Endothelin (1–21) immunoassay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. This assay detects ET-1 and ET-2, but not ET-3 or big endothelin. The detection limit of the immunoassay was 0.05 fmol/ml.

2.6. Immunoblotting

Serum-starved CF were exposed to ET-1 in SFM for 5 min. Whole cell homogenates were prepared and immunoblotting performed using a phospho-specific p44/42 mitogen-activated protein kinase (MAPK) antibody or a p44/42 MAPK expression antibody (loading control), as described previously [22].

2.7. Statistical analysis

All results are expressed as means ± SEM with n representing the number of different patients. Differences between treatment groups were analyzed using paired t tests. Time course and dose response data were compared using repeated measures of one-way analysis of variance (ANOVA) and Newman–Keuls post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ET_AR activation is essential for β_2AR-induced proliferation of human CF

Human CF cultured in MGM (2.5% FCS) supplemented with the β-AR agonist ISO (1 μM) underwent a 53.2 ± 3.2% increase in cell number after 7 days compared with cells grown in MGM alone (Fig. 1A). This was in agreement with our previously published report [5] in which we demonstrated that ISO increased proliferation via activation of the β_2-AR. To investigate whether ET-R activation played a role in this process, we performed proliferation assays in the presence of a non-selective ET-R antagonist (PD142893), or selective antagonists for the ET_A-R (BQ123) or ET_B-R (BQ788). The ISO-induced increase in CF proliferation was fully inhibited by PD142893 and BQ123, but not by BQ788 (Fig. 1A), indicating a role for the ET_A-R in the mechanism of β_2-AR-induced proliferation. None of the ET-R antagonists affected the basal rate of proliferation observed in MGM alone (Fig. 1A).

Our previous work demonstrated that chronic β_2-AR stimulation increased proliferation of human CF via increased secretion of autocrine growth factors [5]. Conditioned medium collected from fibroblasts exposed to MGM containing 1 μM ISO ("ISO-CM") induced a significant increase in proliferation of parallel cultures of CF compared with control CM from cells treated with MGM alone ("C-CM") [5]. Importantly, ISO-CM-induced proliferation occurred in the presence of alprenolol (a β-AR antagonist), confirming that β_2-AR activation is not required for this second phase of the proliferative effect. In the present study, we performed proliferation assays using ISO-CM in the presence of ET-R antagonists to determine whether ET_A-R activation was necessary for the first phase of the proliferative effect (i.e., secretion of autocrine growth factors) or the second phase (i.e., by acting synergistically with the autocrine growth factors to increase proliferation). As shown in Fig. 1B, the ISO-CM-induced increase in cell proliferation was fully inhibited by PD142893 and BQ123, but not by BQ788, in agreement with our data in Fig. 1A. None of the receptor antagonists affected basal proliferation in response to C-CM (Fig. 1B). These data demonstrate that ET_A-R activation is required for the second phase of the proliferative response and is independent of β_2-AR stimulation.

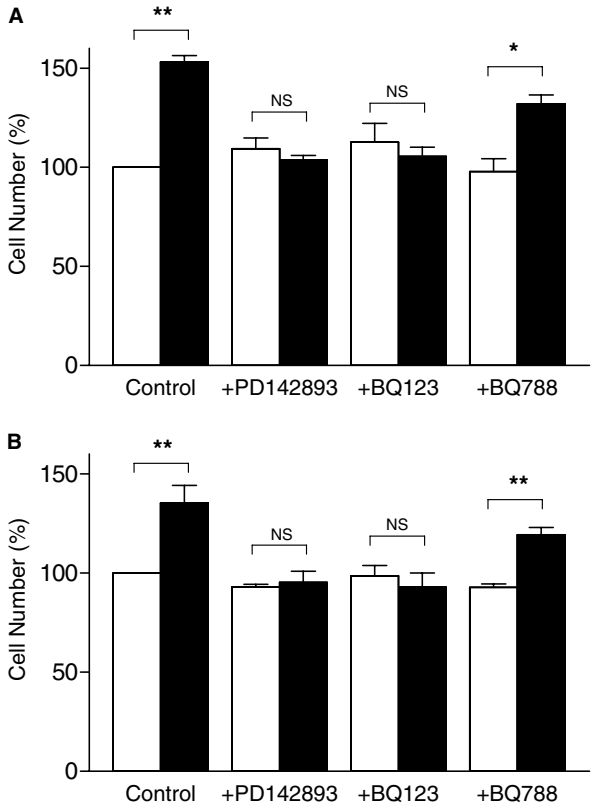

Fig. 1. β_2-AR-induced CF proliferation requires ET_AR activation. (A) CF were exposed to MGM alone (open bars) or supplemented with 1 μM ISO (closed bars) for 7 days with or without 10 μM PD142893, 1 μM BQ123 or 1 μM BQ788. Data are expressed as % cell number observed in MGM control ($n = 4$). $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant for the effect of ISO (paired t test). (B) CF were exposed to C-CM (open bars) or ISO-CM (closed bars) for 7 days with or without PD142893, BQ123 or BQ788. Data are expressed as % cell number observed in C-CM control ($n = 5$). $^{**}P < 0.01$; NS, not significant for the effect of ISO-CM (paired t test).

3.2. Human CF synthesize ppET-1 mRNA and secrete ET-1 independently of β_2-AR stimulation

The initial product of the human ET-1 gene is ppET-1, a 212 amino acid peptide that undergoes sequential enzymatic

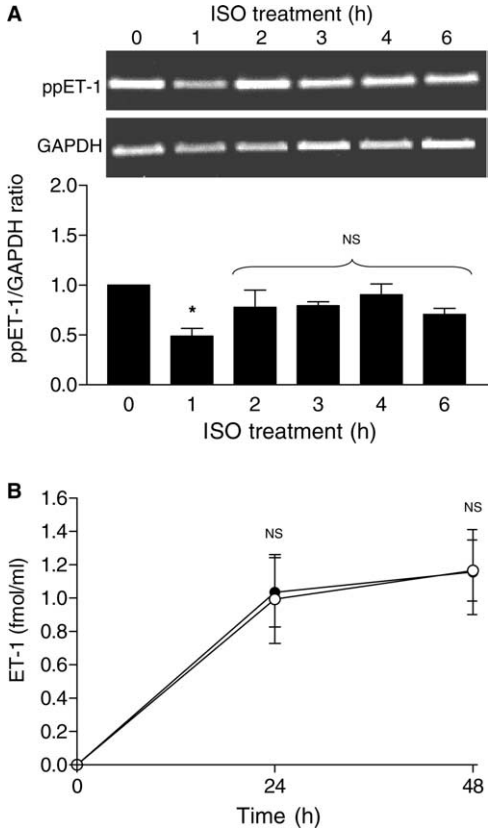

Fig. 2. CF synthesize ppET-1 mRNA and secrete ET-1 independently of β_2-AR stimulation. (A) RT-PCR analysis of ppET-1 mRNA levels following treatment with 1 μM ISO. Bar chart depicts ppET-1/GAPDH ratio after densitometric analysis ($n = 4$). $P < 0.05$ (ANOVA). *$P < 0.05$; NS, not significant for the effect of ISO (Newman–Keuls post-hoc test). (B) Analysis of ET-1 secretion by immunoassay. CF were exposed to MGM alone (open circles) or MGM supplemented with 1 μM ISO (closed circles). NS, not significant for the effect of ISO (paired t test, $n = 5$).

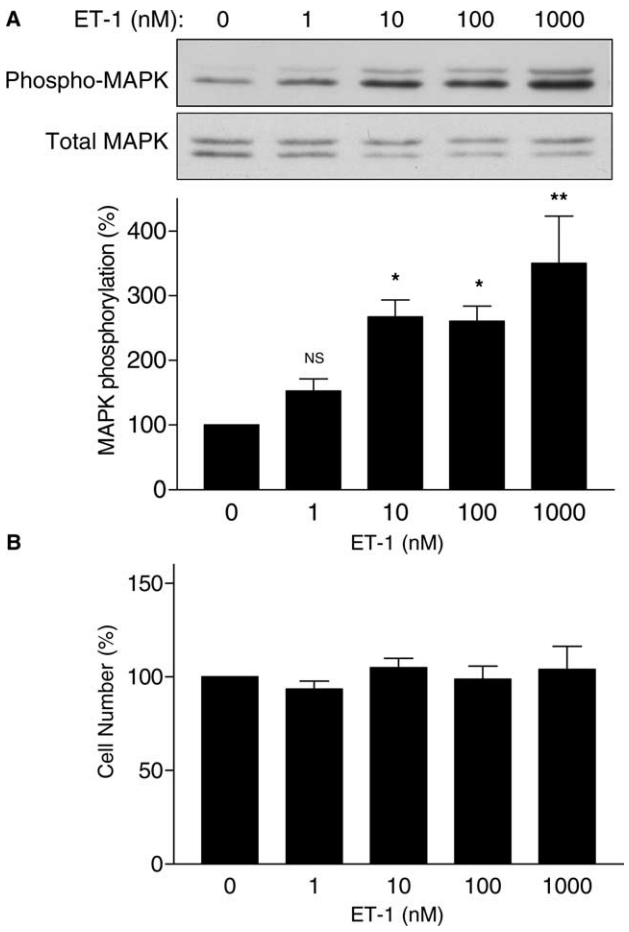

Fig. 3. ET-1 activates p44/42-MAPK but does not increase CF proliferation. (A) Measurement of p44/42-MAPK activation by immunoblotting. CF were exposed to ET-1 for 5 min and homogenates analysed for phosphorylated p44/42-MAPK (upper panel) or total p44/42-MAPK expression (lower panel). Bar chart depicts densitometric analysis of phospho-MAPK ($n = 3$). $P < 0.01$ (ANOVA). **$P < 0.01$; *$P < 0.05$; NS, not significant for the effect of ET-1 (Newman–Keuls post-hoc test). (B) Cell proliferation. CF were exposed to MGM supplemented with ET-1 for 7 days. Data are expressed as % cell number observed in the absence of ET-1 ($n = 4$–7). Not statistically significant (ANOVA).

cleavage to yield ET-1. To determine whether human CF synthesized ppET-1 and whether this was modulated by β_2-AR stimulation, we performed semi-quantitative RT-PCR. In cells exposed to MGM for 24 h, ppET-1 mRNA was readily detectable and levels appeared to decrease slightly over a 6 h period following addition of ISO (Fig. 2A).

To confirm that CF secreted ET-1 peptide, we exposed cells to MGM alone or MGM plus 1 μM ISO for 24–48 h and measured the ET-1 concentration in the medium using an immunoassay. ET-1 was undetectable (i.e., <0.05 fmol/ml) in MGM before it was added to the cells. CF cultured in MGM alone secreted ET-1 with steady-state levels of ~1.1 fmol/ml apparent over the 24–48 h period (Fig. 2B). Supplementation of MGM with ISO did not affect the level of ET-1 secretion (Fig. 2B) regardless of the effects observed on ppET-1 mRNA levels (Fig. 2A).

Taken together, these data demonstrate that human CF synthesize ppET-1 mRNA and secrete ET-1 independently of β_2-AR activation.

3.3. ET-1 activates p44/42 MAPK but does not induce fibroblast proliferation

ET-R stimulation is coupled to activation of the p44/42-MAPK pathway [10]. To confirm that human CF expressed functional ET receptors, we therefore investigated whether exogenously applied ET-1 could stimulate p44/42-MAPK in these cells. As shown in Fig. 3A, ET-1 induced p44/42 MAPK phosphorylation (activation) in a concentration-dependent manner, with maximum stimulation induced by 1 μM ET-1.

To investigate whether ET-1 could increase CF proliferation, we performed proliferation assays in MGM supplemented with ET-1. As shown in Fig. 3B, ET-1 did not stimulate proliferation at any of the concentrations employed. Therefore, although human CF express functional ET-R coupled to p44/42 MAPK activation, this does not result in a proliferative response.

3.4. Role of PKC

As PKC is an important downstream effector of ET-R activation, we next investigated whether PKC activation was involved in the mechanism of ISO-induced CF proliferation.

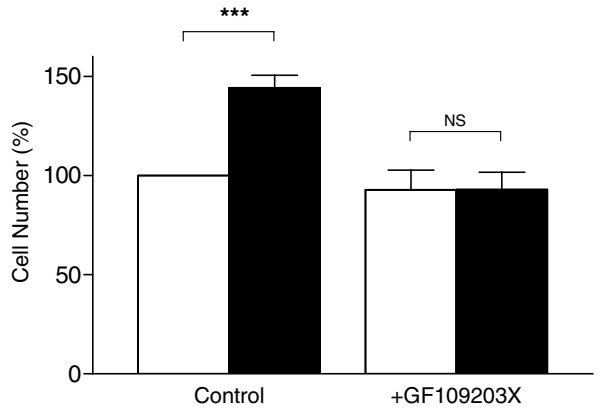

Fig. 4. β_2-AR-induced CF proliferation requires PKC activation. CF were exposed to MGM alone (open bars) or MGM supplemented with 1 μM ISO (closed bars) for 7 days with or without 3 μM GF109203X. Data are expressed as % cell number observed in MGM control ($n = 4$). ***$P < 0.001$; NS, not significant for the effect of ISO (paired t test).

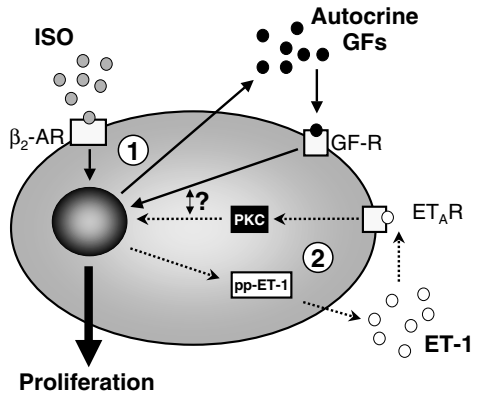

Fig. 5. Proposed role for ET-1 in the mechanism of β_2-AR-induced proliferation of human CF. (1) ISO stimulates the β_2-AR resulting in increased secretion of autocrine growth factors (GFs) that stimulate proliferation via activation of specific growth factor receptors (GF-R). (2) ET-1 is secreted independently of β_2-AR activation and, via activation of the ET_AR and PKC, synergizes with the autocrine growth factors to increase cell proliferation.

The PKC inhibitor GF109203X prevented the ISO-induced increase in cell proliferation, without affecting proliferation in the absence of ISO (Fig. 4), indicating that PKC activation is essential for β_2-AR-induced cell proliferation.

4. Discussion

We have previously reported that chronic β_2-AR stimulation increases proliferation of human CF via a mechanism involving increased secretion of autocrine growth factors [5]. In the present study we extend these observations and provide evidence for an additional autocrine loop in which ET-1 secretion, ET_A-R stimulation and PKC activation play a role. These two distinct phases of the proliferative response are summarized in Fig. 5. Although clearly involved in the ISO-induced proliferative mechanism, ET-1 is not the β_2-AR-induced autocrine factor since (i) ISO treatment does not increase ET-1 secretion (Fig. 2B) and (ii) ET-1 does not increase CF proliferation (Fig. 3B). Thus, ET-1 is as an essential cofactor that acts together with the β_2-AR-induced autocrine growth factors to increase CF proliferation. Studies are currently in progress to identify the autocrine factors which, based on the evidence presented herein, must synergize with ET-1/ET_A-R to induce a proliferative response.

In the present study, we have established that human CF synthesize ppET-1 mRNA and secrete ET-1, in agreement with previous studies using rat CF [13,19,20]. Despite the expression of functional ET-R coupled to activation of the p44/42 MAPK pathway, ET-1 did not increase proliferation of adult human CF under our experimental conditions. Our findings clearly differ from those in neonatal and adult rat [16,17] and embryonic human [23] CF in which ET-1 elicited a proliferative response, and may highlight important interspecies or developmental differences. In our previous study using human CF [5] we also demonstrated that forskolin, an activator of adenylyl cyclase, was unable to induce cell proliferation despite mimicking the effects of ISO on p44/42 MAPK activation. These findings, together with our present data obtained with ET-1, suggest that although p44/42 MAPK activation may be necessary for inducing human CF proliferation [24], its activation alone is insufficient to elicit a proliferative response.

In the normal human left ventricle the ET_A-R accounts for ~60%, and the ET_B-R ~40%, of total ET-R expression [25]. At the cellular level, ET_A-R mRNA expression is localized to myocytes and non-myocytes (predominantly fibroblasts), whereas ET_B-R mRNA is detected almost exclusively in non-myocytes [14]. Combined expression of ET_A-R and ET_B-R has been reported in cultured rat and embryonic human CF [11–13,15], and the ET_B-R may account for as much as 75% of ET-R expression in adult rat CF [11]. It is therefore likely that the cells used in our present study expressed both the ET_A-R and ET_B-R. Although this was not directly investigated, the functional effects of ET-1 in the present study were mediated via the ET_A-R.

A number of animal studies have determined that catecholamine-induced cardiac hypertrophy can be significantly reduced by oral administration of non-selective $ET_{A/B}$ [8] or selective ET_A [9] receptor antagonists. Hence, it was surprising that transgenic mice with cardiomyocyte-specific knockout of the ET_A-R were recently reported to show no reduction in ISO-induced cardiac hypertrophy compared with control mice [26]. However, one explanation offered for this disparity was that the requirement for ET_A-R activation in ISO-induced myocardial remodeling may be at the level of the CF rather than the cardiomyocyte, as ET_AR levels in non-myocytes are not reduced in this model [26]. Our demonstration that the ISO-induced increase in CF proliferation requires ET_AR activation provides corroborative evidence that this may indeed be the case.

In summary, we report here that ET-1, acting via the ET_A-R and PKC activation, is an essential co-factor for β_2-AR-induced proliferation of human CF. These findings provide important new insights into the relationship between the adrenergic and endothelin systems at the level of the CF, and may have implications for the control of adverse myocardial remodeling in man.

Acknowledgements: This work was supported by British Heart Foundation Project Grants PG/02/008 and PG/03/077/15719. We are grateful to Mrs. Jean Kaye and Mrs. Lynne Midgley for excellent technical assistance.

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