Adenylyl cyclase activity and function are decreased in rat cardiac fibroblasts after myocardial infarction

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Running Title: Fibroblast adenylyl cyclase after MI

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Abstract

Myocardial infarction (MI) results in left ventricular remodeling (e.g., ventricular hypertrophy, dilatation and fibrosis). Fibrosis, contributes to increased myocardial stiffening, impaired ventricular filling and function and reduced cardiac output. Adenylyl cyclase (AC) expression and activity are reduced in animal models of heart failure. Stimulation of AC can inhibit extracellular matrix production in isolated cardiac fibroblasts; however, a role for reduced AC expression and activity in fibrosis associated with cardiac remodeling after chronic MI has never been determined. We tested the hypothesis that AC expression and activity are reduced in cardiac fibroblasts after chronic (18 week) MI. Rats underwent coronary artery ligation or sham surgery (Con) and echocardiography was used to assess left ventricular remodeling 1, 3, 5, 7, 10, 12 and 18 weeks after surgery. Cardiac fibroblasts were isolated from the non-infarcted myocardium and compared for differences in AC activity and collagen synthesis. End-diastolic dimension was increased (Con: 0.76 ± 0.02 (SEM) cm, MI: 1.0 ± 0.02 cm; p<0.001) and fractional shortening decreased (Con: 44 ± 2%, MI: 17 ± 2%; p<0.001) in MI compared to control rats. Basal and forskolin-stimulated cAMP production were decreased by 90% and 93%, respectively, and AC5/6 expression was decreased 39% in fibroblasts isolated from MI rats compared to sham controls. Serum-stimulated collagen production was increased 2-fold and forskolin-mediated inhibition of collagen synthesis was reduced in fibroblasts from MI rats compared to controls. Our data demonstrate that AC expression and activity are reduced and collagen production is increased in cardiac fibroblasts of rats after MI.
Introduction

Congestive heart failure (CHF) is a leading cause of morbidity and mortality in the U.S. CHF was believed to result primarily from systolic dysfunction; however, it is recognized that diastolic dysfunction, or an inability of the heart to fill during diastole, also is an underlying mechanism in many patients with heart failure (29). Myocardial fibrosis, a key contributor to cardiac dysfunction during CHF, reflects hyperplasia and increased deposition of extracellular matrix (ECM) material into the interstitial and perivascular space (5). Exaggerated ECM deposition results in myocardial stiffening and decreased relaxation of the heart, ultimately leading to cardiac dysfunction (19, 20).

The myocardium is composed of cardiac myocytes and non-myocytes, which include endothelial cells, vascular smooth muscle cells and fibroblasts (34). Cardiac fibroblasts (CF), an abundant cell type in the heart (comprising ~ 2/3 of the total cell population) are responsible for basal ECM homeostasis as well as repair after a cardiac insult (7, 19). Following myocardial infarction (MI), reparative scar formation at the site of injury can initiate maladaptive connective tissue production remote from the infarct zone in the remaining functional regions of the myocardium (1, 21, 31, 33). This increased extracellular matrix contributes to the impaired cardiac compliance, reduced filling capacity, and cardiac dysfunction involved in heart failure.

Adenylyl cyclases (AC), membrane-bound enzymes that catalyze the conversion of ATP to cyclic AMP (cAMP), are activated upon stimulation of G protein-coupled receptor (GPCR) agonists that signal through G_{aS}. Of the nine membrane-bound AC isoforms, adenylyl cyclase type 5 (AC5) and type 6 (AC6) are the most highly expressed isoforms in the heart and have direct effects on cardiac function (9, 11, 18). Interestingly, AC6 mRNA expression and total AC activity are reduced in animal models of heart failure (26) and overexpression of AC6 attenuates
deleterious remodeling and increases function in the heart (17, 27, 28). Our laboratory and others have shown that AC6 activation and expression and increased cAMP production inhibit fibroblast proliferation and collagen synthesis, suggesting an anti-fibrotic role for AC6 in the heart (4, 6, 12, 24, 32). However, a role for AC expression and activity in fibrosis associated with cardiac remodeling after chronic MI has never been established.

We hypothesized that AC activation negatively regulates collagen production in the heart and that downregulation of AC expression and activity following MI may contribute to cardiac fibrosis. To test this hypothesis we conducted echocardiographic assessment of left ventricular (LV) function over an 18-week period after MI or sham surgery and analyzed changes in AC expression and activity and collagen production in adult rat CF isolated from the non-infarcted regions of the LV. We show that total AC activity and AC5/6 expression are reduced concomitant with increased collagen production in MI rats compared to sham controls. These data suggest that a downregulation of AC function in the heart after MI may exacerbate maladaptive connective tissue production thereby contributing to cardiac fibrosis and heart failure.

**Materials and Methods**

**Animals.** Animals were handled in accordance with the animal welfare regulations of the VA San Diego Healthcare System and NIH guidelines. Adult male (200 – 250 g) Sprague Dawley rats were used. Twenty-five rats underwent thoracotomy and coronary artery ligation (MI group) and 8 rats underwent thoracotomy and no coronary artery ligation (Sham control group). All animals were killed 18 weeks after surgery.
**Myocardial Infarction.** Anesthetic induction was accomplished using isoflurane (5% in 100% O₂). Rats were intubated using a 16 gauge angiocatheter and mechanically ventilated with 1.5% isoflurane in 100% O₂ to maintain anesthesia. Animals were placed in the left lateral decubitus position and the chest cavity was opened at the third intercostal to visualize the heart. The left coronary artery was ligated approximately 2 mm below the edge of the left atrial appendage by placing a 7-0 prolene suture (tapered needle) around the artery. LV blanching indicated successful occlusion of the vessel. The chest and skin were closed with suture (6-0 nylon) and the rats allowed to recover.

**Echocardiography.** Echocardiography was performed in anesthetized rats prior to surgery and at 1, 3, 5, 7, 10, 12, 14 and 18 weeks post-surgery. Rats were anesthetized using isoflurane anesthesia (1.5% in O₂) supplied via a nose mask. Using a pediatric 12 MHz linear probe (Agilent Technologies) a parasternal short axis view was obtained as a guide for LV M-mode imaging at the papillary muscle level. The M-mode images were digitized on the optical disc (HP 5500). Using the HP 5500 standard software, LV dimensions were traced in both end-diastole and end-systole in short and long axis views and LV volumes determined using the modified Simpson method and percentage fractional area change (%FS) was calculated. Infarct size was assessed by measuring regional wall motion in both long axis (4 regions, anterior septal, anterior apical, posterior septal and posterior apical segments) and short axis views (4 regions, anterior, lateral septal and inferior wall segments) and assigning an echo score of 1 point for akinesis or dyskinesis in any region (2). Based on these measurements rats with an echo score ≥ 4 (18-week echo) were included in the infarct group.
**Isolation and Culture of Adult Rat CF.** Eighteen weeks after MI, CF were isolated from the non-infarcted LV of MI rats or from the LV septa of control groups as previously described (32). All CF were used at early passage (≤ 2) to minimize loss of the *in vivo* phenotype as a result of cell division *in vitro*. Homogeneity of the cell preparation was verified by positive staining for fibroblast specific markers: discoidin domain receptor 2 (DDR2) and fibroblast specific protein 1 (FSP1). Immunohistochemical staining and image acquisition were performed as previously described (32).

**Collagen Synthesis Assay.** Collagen synthesis was measured using a collagenase-sensitive [*³H*]-proline incorporation according to previously established methods (25, 32).

**Cyclic AMP Production.** cAMP production by CF was measured according to previously described methods (32).

**Immunoblot Analysis.** Antibody for AC type 5/6 was obtained from Santa Cruz Biotechnology. Immunoblot analysis was conducted as previously described (32).

**Data analysis.** Statistical comparisons and graphical representation were performed using GraphPad Prism 3.0 (GraphPad Software). ANOVA was followed by post-hoc Bonferroni correction for multiple comparisons. Statistical significance was set at *p*<0.05.

**Results**
Echocardiographic assessment of LV size and function after MI. Compared to control animals, rats with MI exhibited increased LV chamber diameter and thinning of the anterior LV freewall, indicative of myocardial tissue loss in the infarct (Figures 1A and B). LV remodeling post-MI was associated with a significant (p<0.05) decrease in % fractional shortening (%FS; Figure 1C) and increased end-diastolic dimension (EDD; Figure 1D), demonstrating reduced cardiac function and progressive LV chamber dilatation after MI. No significant changes in %FS or EDD were observed in control rats.

AC activity of CF is reduced following MI. Homogeneity of the cell preparation from control and MI rats was confirmed by positive staining with DDR2 and FSP1, specific markers of fibroblasts (Figure 2A). No observable contamination of other cell types was present. In order to analyze differences in AC activity by CF isolated post-MI, cAMP production was measured under basal conditions and in response to the direct AC agonist, forskolin (Fsk; 10 µM). Although no differences in basal cAMP production were observed, there was a 93% reduction (p<0.05) in Fsk-stimulated cAMP production by CF from MI rats compared to controls (Figure 2B).

AC expression is reduced following myocardial infarction. In order to determine whether decreased cAMP production by CF results from reduced AC expression, we examined expression of AC 5/6, the predominant AC isoforms expressed in the heart (9, 11, 23), using immunoblot. AC 5/6 expression was significantly (p<0.05) reduced in CF from MI rats compared to controls (Figure 3).
Collagen production by CF is increased following MI. Because AC activation exerts anti-fibrotic effects on fibroblasts in culture (4, 6, 12, 24, 32), we hypothesized that the decreased AC expression and activity may correlate with increased collagen production by CF following MI. Consistent with this hypothesis, CF from MI rats exhibited enhanced (p<0.05) serum-stimulated collagen production (as indicated by $[^3]$H-proline incorporation) compared to control CF (Figure 4). In contrast, forskolin reduced (p<0.05) serum-stimulated collagen production to basal levels in control CF, whereas CF from infarcted rats exhibited only moderate inhibition in response to forskolin treatment. Thus, increased collagen production by CF following MI may result from decreased AC expression and a decreased ability of CF to produce cAMP.

Discussion

We determined whether the fibrosis and connective tissue production involved in cardiac remodeling after chronic MI results, at least in part, from a downregulation of AC. Previous studies have demonstrated AC6 mRNA expression and total AC activity are reduced following pacing-induced heart failure in pigs (26). However, no previous studies have examined AC function and collagen production by cardiac fibroblasts isolated from the heart 18 weeks after myocardial infarction. We provide the first evidence that AC activity and expression by cardiac fibroblasts is reduced in hearts from rats after chronic MI. Furthermore, we show that decreased AC function correlates with increased collagen production by cardiac fibroblasts.

Several in vitro studies have demonstrated that AC can have anti-fibrotic effects, whereby G protein-coupled receptor (GPCR) agonists that activate AC and stimulate cAMP production can inhibit collagen synthesis by fibroblasts from various organs in culture (4, 6, 12, 24). Data from our laboratory has shown that CF overexpressing AC6 exhibited a greater
inhibition of TGFβ-stimulated myofibroblast formation and serum-stimulated collagen production as well as decreased basal and TGFβ-promoted expression of the pro-fibrotic factors PAI-1 and IL-6 (32). Thus, cardiac fibrosis following MI may be potentiated by a downregulation in AC thereby reducing the ability of AC to negatively regulate collagen production by CF after MI. Antifibrotic effects of AC in isolated cells suggest that AC may have a therapeutic role in post MI remodeling. It is known that animals post-MI have increased sympathetic drive (10); in this regard others have shown decreased cardiac AC expression in animal models of heart failure (8, 13, 26). Our results suggest the increased sympathetic tone post-MI may be responsible for the decreased expression and activity of AC in cardiac fibroblasts and contribute to fibrosis in the non-infarcted myocardium. Our findings have to be considered in the context of other findings that suggest that decreased expression of AC5 in cardiac myocytes may decrease beta-adrenergic receptor stimulated apoptosis and preserve cardiac function in the cardiac remodeling associated with pressure overload hypertrophy (14, 22).

A limitation of the current study is the potential loss of the pro-fibrotic phenotype by CF that are removed from the in vivo cardiac milieu and studied under in vitro culture conditions. For this reason, we conducted all experiments using low passage cells (pass ≤ 2) to avoid any de-differentiation of the CF. In an MI model, previous studies have shown that the in vivo cardiac fibroblast phenotype is stable and can be studied in culture up to passage 4 (32). The methodologies presented here are consistent with previous reports examining protein expression and signaling by CF isolated from rats after MI and studied in culture (3, 15, 16, 30). However, unlike these studies which examined CF at early time points (≤ 4 weeks) post-MI, we examined the long term effects of MI on AC function and collagen production to understand the role of AC
in cardiac fibroblast function in the late phase of remodeling. The progressive fibrosis in the non-infarcted myocardium that leads to diastolic dysfunction in mice occurs much later, reaching a maximum at approximately 4 months post-MI (35).

In conclusion, our data provide new information regarding AC expression and activity in cardiac fibroblasts after MI. These results suggest a potential role for AC as an anti-fibrotic mediator of the deleterious connective tissue remodeling that occurs late after MI.
Figure Legends

Figure 1. Cardiac size is increased and cardiac function is reduced in adult rat CF 18 weeks after myocardial infarction. M-mode echocardiographic images were obtained after infarct (MI) or sham surgery (control). Arrows denote the distance between the anterior LV freewall and the posterior LV (septum) during diastole. (C) % fractional shortening (%FS) and (B) end-diastolic diameter (EDD) were measured before and 1, 3, 5, 7, 10, 12, 14, and 18 weeks after myocardial infarction or sham-surgery to assess LV function and dilatation. Values represent mean ± SEM of at least 5 experiments compared using Two-way analysis of variance with post-hoc multiple comparisons test. p<0.05 denotes control vs. MI rats.

Figure 2. cAMP production is reduced in adult rat CF 18 weeks after myocardial infarction. A. Homogeneity of cell preparation was confirmed by DDR2 and FSP1 staining. B. cAMP production by CF isolated from the non-infarcted LV region of rats 18 weeks after infarct (MI) or sham surgery (control). cAMP production was measured by radioimmunoassay using CF grown for 48 hr in serum-free media and then stimulated for 10 min with 2.5% fetal bovine serum (FBS) alone (basal) or in the presence of forskolin (Fsk, 10 µM). Values represent mean ± SEM of n = 5-7 rats per group. * denotes p<0.05 compared to control using a Two-way analysis of variance with post-hoc multiple comparisons test.

Figure 3. AC5/6 expression is reduced in adult rat CF 18 weeks after myocardial infarction. AC 5/6 expression was measured by immunoblot using CF (passage ≤2) isolated from the non-infarcted LV region of rats 18 weeks after infarct (MI) or sham surgery (control). AC5/6 immunoreactive bands were normalized to that of GAPDH and data are expressed as the mean ±
SEM of n = 5-6 rats per group. * denotes p<0.05 compared to control compared using an unpaired Student’s t-test.

*Figure 4. Collagen production by CF is increased following MI.* Collagenase-sensitive $[^3]H$-proline incorporation by CF isolated from the non-infarcted LV region of rats 18 weeks after infarct (MI) or sham surgery (control). CF were grown for 48 hr in serum-free media and then stimulated for 48 hr with 0% fetal bovine serum (control) or 2.5% in the absence or presence of forskolin (Fsk, 10 µM). Data are normalized for $[^3]H$-proline incorporation into cells grown under control conditions. Values represent mean ± SEM of n = 5-6 rats per group. * denotes p<0.05 compared to control and # denotes p<0.05 compared to 2.5% FBS alone using Two-way analysis of variance with Bonferroni post-hoc multiple comparisons tests.
References


8. Espinasse I, Iourgenko V, Richer C, Heimburger M, Defer N, Bourin MC, Samson F, Pussard E, Giudicelli JF, Michel JB, Hanoune J, and Mercadier JJ. Decreased type VI adenylyl cyclase mRNA concentration and Mg(2+)-dependent adenylyl cyclase activities and unchanged type V adenylyl cyclase mRNA concentration and Mn(2+)-dependent adenylyl...


Figure 1

A. 

B. 

C. 

D. 

230x171mm (72 x 72 DPI)
Figure 2

A

Control

MI

B

cAMP production (pmol/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MI</th>
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</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fsk</td>
<td>4000</td>
<td>3000*</td>
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159x177mm (300 x 300 DPI)
Figure 3

AC5/6

GAPDH

Relative AC 5/6 expression

Control | MI

138x162mm (72 x 72 DPI)
Figure 4

[Insert bar graph showing [3H]-Proline Incorporation (fold over basal) for control and Fsk treated conditions, with statistical significance indicated by asterisks (*) and hash (#).]

+ 2.5% FBS

192x169mm (72 x 72 DPI)