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EPAC expression and function in cardiac fibroblasts and myofibroblasts

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ABSTRACT

In the heart, cardiac fibroblasts (CF) and cardiac myofibroblasts (CMF) are the main cells responsible for wound healing after cardiac insult. Exchange protein activated by cAMP (EPAC) is a downstream effector of cAMP, and it has been not completely studied on CF. Moreover, in CMF, which are the main cells responsible for cardiac healing, EPAC expression and function are unknown. We evaluated in both CF and CMF the effect of transforming growth factor β1 (TGF-β1) on EPAC-1 expression. We also studied the EPAC involvement on collagen synthesis, adhesion, migration and collagen gel contraction.

Method: Rat neonatal CF and CMF were treated with TGF-β1 at different times and concentrations. EPAC-1 protein levels and Rap1 activation were measured by western blot and pull down assay respectively. EPAC cellular functions were determined by adhesion, migration and collagen gel contraction assay; and collagen expression was determined by western blot.

Results: TGF-β1 through Smad and JNK significantly reduced EPAC-1 expression in CF, while in CMF this cytokine increased EPAC-1 expression through ERK1/2, JNK, p38, AKT and Smad3. EPAC activation was able to induce higher Rap1-GTP levels in CMF than in CF. EPAC and PKA, both cAMP effectors, promoted CF and CMF adhesion on fibronectin, as well as CF migration; however, this effect was not observed in CMF. EPAC but not PKA activation mediated collagen gel contraction in CF, while in CMF both PKA and EPAC mediated collagen gel contraction. Finally, the EPAC and PKA activation reduced collagen synthesis in CF and CMF.

Conclusion: TGF-β1 differentially regulates the expression of EPAC in CF and CMF; and EPAC regulates differentially CF and CMF functions associated with cardiac remodeling.

Introduction

Cardiac fibroblasts (CF) are the major non-myocyte cell constituent in the myocardium and actively participate in the maintenance of myocardial structure by controlling the homeostasis of the extracellular matrix (ECM) in normal tissue (Mc Anulty, 2006; Porter and Turner, 2009). CF are differentiated to cardiac myofibroblasts (CMF), by transforming growth factor β1 (TGF-β1), and these differentiated cells are actively involved in wound healing after cardiac injury and in tissue remodeling (Tomasek et al., 2002; Van den Borne et al., 2009). We have shown that the β2-adrenergic receptor (β2-AR) is the most expressed β-AR subtype in CF and CMF (Aránguiz-Urroz et al., 2011). Indeed, β-ARs have been identified on both neonatal and adult rat CF, and the stimulation with β-adrenergic agonists promotes proliferation, reduces collagen secretion (Copaja et al., 2008; Ocaranza et al., 2002; Sun and Weber, 2005), and induces autophagy (Aránguiz-Urroz et al., 2011).

EPACs exert their function on GTPases Rap1 and 2, and both regulate many biological functions that are cell-specific such as proliferation, survival and differentiation, as well as intercellular adhesion, protein secretion and ion transport (Bos, 2006). Experiments performed on mice hearts demonstrated that EPAC-1/EPAC-2 mRNA levels decrease with heart development. Furthermore, it was shown that in mice with cardiac hypertrophy, EPAC-1 expression levels were increased, and the cAMP–EPAC–Rap1 signaling pathway was functioning, which suggests EPAC-1 participation in the cardiac hypertrophic process (Ulucan et al., 2007). Data presented by Yokoyama et al. showed that CF have higher EPAC-1 protein expression levels than EPAC-2 protein; and that TGF-β1, a cytokine closely related to cardiac fibrosis, was able to promote only the decrease in EPAC-1 mRNA and protein expression levels in CF (Yokoyama et al., 2008). In addition, it has been observed that EPAC participates in collagen synthesis. EPAC through PI3K signaling pathway activation might be related to the decrease in collagen synthesis in CF induced by adenosine agonists (Villarreal et al., 2009).

Despite the aforementioned background, the TGF-β1 effects on EPAC-1 protein levels in CF and mainly in CMF are unknown. Also, it is unknown the signaling pathways activated by TGF-β1 which is

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involved in the control of EPAC-1 expression in both cell types. The knowledge regarding EPAC function in CF and CMF and how this protein could participate in process associated to cardiac remodeling such as collagen synthesis, migration, adhesion and collagen gel contraction still remained to be investigated. The results presented in this paper suggest that TGF-β1 differentially regulates EPAC-1 protein levels in CF and CMF, and moreover, present new data related to the potential role of these cells in wound healing.

Methods

Reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): trypan blue, 8-(4-chlorophenylthio)-2-O-methyladenosine-3,5-cyclic monophosphate (Me-cAMP), N6-phenyladenosine-3,5-cyclic monophosphate (6-Bz-cAMP), antibodies to α-smooth muscle actin and β-tubulin, ERK inhibitor (PD98059), JNK inhibitor (SP600125), p38 inhibitor (SB202190) and ALK5 inhibitor (SB431542). Trypsin/EDTA, prestained molecular weight standard, fetal bovine serum (FBS) and fetal calf serum (FCS) were from GIBCO BRL (Carlsbad, CA, USA). Smad3 inhibitor (SiS3) and all organic and inorganic compounds were purchased from Merck (Darmstadt, Germany). The enhanced chemiluminescence (ECL) reagent was acquired from Perkin Elmer Life Sciences, Inc. (Boston, MA, USA). Sterile plastic material was purchased from Falcon® (NJ, USA). The primary antibodies for EPAC-1, phospho-Smad2, phospho-ERK, phospho-JNK, phospho-p38, total-ERK, total-JNK, total-p38 and total-Smad2 were purchased from Cell Signaling Technology (Boston, MA, USA). The rabbit and mouse secondary antibodies were acquired from Calbiochem (Darmstadt, Germany). Accutase®, TGF-β1 and pro-collagen antibody were purchased to Chemicon (Temecula, USA). Rat tail type I collagen was obtained from Santa Cruz Biotechnology Inc. (California, USA).

Animals

Rats were obtained from the Animal Breeding Facility of the School of Chemical and Pharmaceutical Sciences at the University of Chile. All studies were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and experimental protocols were approved by our Institutional Ethics Review Committee.

Isolation and culture of ventricular cardiac fibroblasts of neonate rats

CF were isolated from neonate Sprague–Dawley rats as previously described (Diaz-Araya et al., 2003). Neonate rats were decapitated and their hearts were carefully extracted. Isolated ventricles were then incubated in pancreatin and collagenase II solutions for further digestion. The homogenized tissue was placed into a 100-mm dish and allowed to adhere to the dish for 2 h at 37 °C in culture media containing 5% FBS and 10% FCS. During this process CF were separated from cardiomyocytes by differential adhesion to the dish. The CF were then cultured in DMEM-F12 + 10% FBS until 80% confluence. Cells were washed twice with phosphate buffer saline (PBS), trypsinized, and passaged twice over a 5-day period. After passage 2 fibroblasts were maintained with DMEM-F12.

Differentiation from cardiac fibroblasts to cardiac myofibroblasts

In our laboratory CF to CMF differentiation is a well characterized cellular process. Primary cultures of CF were serum starved for 24 h, and then stimulated once with TGF-β1 (5 ng/mL) up to 96 h (time for complete differentiation). Cell culture was characterized as CMF (α-SMA positive). After treatment period (96 h), CMF were washed twice with PBS 1× to withdraw TGF-β1 remnant and then cultivated for 24 h in fresh DMEM-F12. Then CMF were washed again with PBS 1× and the stimuli (either TGF-β1; as new stimuli, or another one i.e. Me-cAMP) were added in fresh media. The cells remained at 37 °C and 5% CO_2 for 24 h or 48 h depending on the experiments.

Western blot of proteins in fibroblast and myofibroblast cultures

Proteins extracts were separated by 8% polyacrylamide gel electrophoresis. For western blots 50 μg of total proteins was used. SDS-PAGE was performed at 100 V in 1× electrophoresis buffer (Tris Base 30.25 g, Glycine 144 g, SDS 10 g, water 1 L for a 10× electrophoresis buffer). Proteins were electrotransferred to a nitrocellulose membrane (BioRad) at 350 mA over 120 min in transference buffer. Membranes were blocked using blocking buffer (non-fat milk 5% (w/v)/TBS-1×–Tween 0, 1%) during 60 min at room temperature. After blocking three wash cycles were performed using TBS 1×–Tween 0, 1%. Membranes were incubated with the antibodies overnight at 4 °C in a dilution of 1:1000 or 1:5000 depending on antibody type. After incubation, membranes were washed three times with TBS 1×–Tween 0, 1% for 10 min. Secondary antibody was incubated for 2 h at room temperature using a 1:5000 dilution. After washing with TBS 1×–Tween 0, 1% the membranes were exposed to ECL reagent. BioMax Film was used for western blot registering. Blots were quantified by densitometry. Results were expressed as the ratio of protein in experimental conditions versus control samples. Tubulin was used as loading control.

Rap1 pull-down assay

Rap1 activity was measured using Rap1 activating assay kit (Cell Biolabs Inc.) according to the manufacturer's recommendations. Briefly CF and CMF (70%–80% confluent) grown in 100-mm dishes were treated with an EPAC selective cAMP analog (Me-cAMP). After stimulation, cells were lysed on ice using the manufacturer's lysis buffer. Cell lysates were homogenized by sonication and cell debris was removed by a 5 min centrifugation at 3000 *g* at 4 °C. The supernatants were incubated with GST-RBD beads for 1 h at 4 °C on a rotator. Beads were washed and then immunoprecipitated. The beads were suspended in 2× SDS sample buffer and subjected to 15% SDS-PAGE followed by western blot analysis.

Cell adhesion assay

CF and CMF were maintained in serum free conditions and fresh medium 24 h before the adhesion assay. Cells were incubated with isoproterenol, forskolin, Me-cAMP and 6-Bz-cAMP, and then were detached by trypsinization. Cells were counted and suspended at 20,000 cells/mL in DMEM-F12 containing 10% FBS and seeded in a 24-well plate, which was coated overnight with fibronectin (100 μg/mL). Adhesion was allowed to proceed for 2 h at 37 °C and non-adherent cells were removed by gently washing the plates three times with warmed PBS 1×. Adherent cells were stained using crystal violet at room temperature for 20 min, lysed in the wells by adding 200 μL of SDS 10%, and quantified spectroscopically at 595 nm.

Migration assays

Scratch assay. Cells were cultured to confluence and maintained in serum free conditions for 24 h before the experiments. A scratch was made in CF and CMF monolayers with a blue pipette tip and after 1 h cells were stimulated with isoproterenol, forskolin, Me-cAMP and 6-Bz-cAMP. After 48 h cells were stained with crystal violet for 20 min at room temperature. Images were obtained with a microscope. Images shown in figure are representative of four independent

experiments. Data was expressed as percent of reduction of the scratched area.

Costar Transwell system. The Costar Transwell system (Corning Life Sciences, Acton, MA, USA) allows cells to migrate through an 8 mm pore size in a polycarbonate membrane. Briefly, pretreated cells were trypsinized, washed, and suspended in serum-free DMEM-F12 (300,000 cells/mL). This suspension (100 μL) was added into the Transwell upper chamber. The lower chamber was filled with 500 μL DMEM-12 containing 10% FBS as chemoattractant. After 2 h stimulation, chambers were removed, and the cells remaining on the upper surface of the membrane were removed with a cotton swab. Later, membranes were washed with PBS 1×, and cells that adhered on the lower surface of the membrane were fixed with cold methanol for 15 min and stained with crystal violet. Cells were counted in 40 high-power microscope fields. For each experiment 12 images per condition were taken, and each experiment was repeated 4 times.

Gel collagen contraction

A solution of rat tail collagen type I (3.63 mg/mL, Santa Cruz Biotechnology Inc.) was mixed with DMEM containing 10 mM Hepes buffer (GIBCO, Grand Island, NY) to maintain neutral pH, 100,000 cells/mL (detached by trypsin from monolayer confluent cultures); and appropriate dilutions of isoproterenol, forskolin and cAMP analogs. The individual solutions were prepared and cooled to 4 °C before mixing to prevent premature gelification. In a 24-well plate the mix was incubated for 2 h at 37 °C for collagen polymerization. Thereafter, the gels were detached from the wells and 48 h later images were taken in order to evaluate the reduction in gel collagen area.

Statistical analysis

Data are expressed as the mean ± S.E.M. of *n* independent experiments. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism Software. $p < 0.05$ was considered significant.

Results

Expression levels of EPAC-1 in cardiac fibroblasts and myofibroblasts

As shown in Fig. 1A, TGF-β1 (5 ng/mL, for 96 h) increased the expression levels of α-SMA (a marker of CF to CMF differentiation), this result is showing that CF were effectively differentiated to CMF. Meanwhile, in Fig. 1B, we show that EPAC-1 levels decreased significantly 24 h after the treatment with TGF-β1 (5 ng/mL). However, the EPAC-1 levels were recovered and, as shown in the same figure, at 96 h they reached levels significantly higher than those observed in CF. By contrast, TGF-β1 (5 ng/mL) significantly increased EPAC-1 levels in CMF after 24 h and 48 h of treatment (Fig. 1C). These results suggest that TGF-β1 differentially regulates EPAC-1 levels in CF and CMF.

When CF or CMF were stimulated with different concentrations of TGF-β1 for 24 h, only at higher concentrations (5 and 10 ng/mL), EPAC-1 protein levels were significantly decreased in CF (Fig. 1D), whereas in CMF, the same concentrations of TGF-β1 for the same incubation period significantly increased EPAC-1 levels (Fig. 1E). On the other hand, our results (Supplementary Fig. 1B), showed that repetitive stimulation of CF (one daily dose, starting at 0, 24, 48 and 72 h) with TGF-β1 (5 ng/mL) maintains the EPAC-1 protein levels decreased during 48 h. However, at 72 h there were no significant differences with respect to control levels, and later (96 h) EPAC-1 levels were increased similar to those levels found in CMF (CF treated once with TGF-β1 by 96 h). While, in CMF, our results (Supplementary Fig. 1D) showed that repetitive stimulation (one daily dose, starting at 0, 24 and 48 h) with TGF-β1 (5 ng/mL) maintains the EPAC-1 levels increased during 48 h.

TGF-β1 modulates EPAC-1 expression levels by different signaling pathways in cardiac fibroblasts and myofibroblasts

It is well-known that TGF-β1 signals through the activation of Smads (canonical pathway), as well as the activation of MAPKs (ERK1/2, JNK and p38). Thus, we first evaluated if TGF-β1 was able to activate both Smad and MAPK pathways in CF and CMF. Supplementary Figs. 2A–B show that this cytokine activates the Smad pathway in CF and CMF, respectively. Similarly we demonstrated that TGF-β1 significantly activates ERK1/2, JNK and p38 both in CF (Supplementary Figs. 2C, E and G) and in CMF (Supplementary Figs. 2D, F and H). In order to confirm the specificity of the chemical inhibitors on each signaling pathway, CF were pre-treated with PD98059 (ERK1/2 inhibitor), SP600125 (JNK inhibitor), SB202190 (p38 inhibitor) or SB431542 (TGF-β1 type I receptor inhibitor) and then phosphorylation levels were measured. As expected, in Supplementary Figs. 3A–D, it can be seen that the inhibitors blocked the phosphorylation of ERK1/2, JNK, p38 and Smad2 after TGF-β1 treatment, respectively.

To identify which of the above mentioned pathways was participating on the observed changes in EPAC-1 levels, CF or CMF were treated with TGF-β1 (5 ng/mL) for 24 h, in the presence of each inhibitor. As observed in Fig. 2A the decrease in EPAC-1 levels induced by TGF-β1 in CF was blocked by SP600125 and SB431542, but not by PD98059 or SB202190. In contrast, all inhibitors for both canonical and MAPK pathways prevented the increase in EPAC-1 levels induced by TGF-β1 in CMF. To assess if Smad3 as well as PI3K–Akt were participating in EPAC-1 regulation in CF and CMF, we studied EPAC-1 protein levels in the presence of the inhibitors SIS3 (Smad3) and LY294002 (Akt). The results showed that Smad3 and PI3K–Akt are only involved in the regulation of EPAC-1 in CMF, but not in CF (see Supplementary Figs. 4A–D).

cAMP–EPAC1–Rap1 activity in cardiac fibroblast and myofibroblast

Because EPAC-1 levels are significantly higher in CMF than CF, we studied the activation levels of the downstream effector of EPAC, Rap1. The overall Rap1 levels can be observed in Fig. 3A, and the results did not show significant difference between CF and CMF. However, the levels of Rap1-GTP, once stimulated with an EPAC selective cAMP analog (Me-cAMP) were compared, and Fig. 3B shows that Rap1-GTP levels were higher in CMF than in CF.

EPAC regulates differentially migration, adhesion, contraction of collagen gels and collagen synthesis in cardiac fibroblast and myofibroblast

Adhesion studies were performed with CF and CMF, which were stimulated with two compounds able to increase intracellular cAMP (isoproterenol, forskolin), and selective cAMP analogs for EPAC (Me-cAMP) or PKA (6-Bz-cAMP). CF stimulated with isoproterenol, forskolin, and Me-cAMP but not with PKA selective cAMP analog showed significantly greater adhesion to fibronectin compared to control (Fig. 4A). Similarly, Fig. 4B shows that isoproterenol, forskolin and Me-cAMP were also able to significantly increase the adhesion to fibronectin in CMF when compared to control. In Figs. 5A–B, migration was quantified in CF and CMF using the scratch method. Isoproterenol, forskolin and Me-cAMP, but not 6-Bz-cAMP, were able to significantly promote migration of CF compared to control, while no effect was observed in CMF (see Supplementary Figs. 5A–B). To quantify the effect of the aforementioned stimulus on CF migration, experiments were performed in Transwell chambers. The results obtained

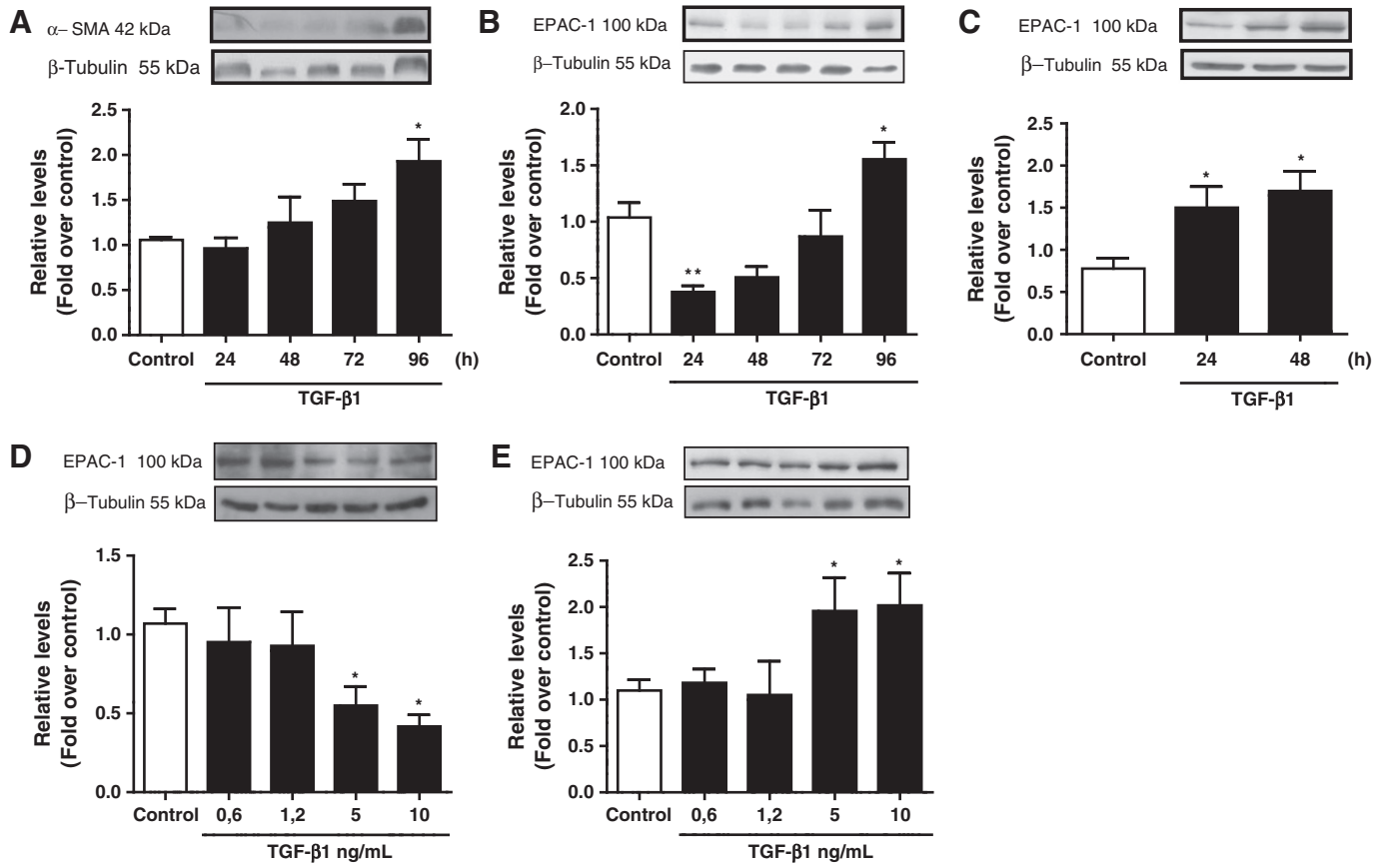

Fig. 1. TGF-β1 regulates EPAC-1 expression in cardiac fibroblasts (CF) and myofibroblasts (CMF). (A) TGF-β1 (5 ng/mL), in a time dependent manner increased α-SMA expression in cardiac fibroblasts. (B and C) TGF-β1 regulates differentially EPAC-1 expression in CF and CMF, respectively. (D) TGF-β1 decreased in a concentration dependent manner EPAC-1 expression levels in CF. (E) TGF-β1 increased in a concentration dependent manner EPAC-1 expression levels in CMF. In each experiment, protein levels were assessed by western blot; tubulin was used as load control. A representative immunoblot is shown as well as their respective graphic analysis. The results are means (±S.E.M.) of 6 independent experiments. ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs control).

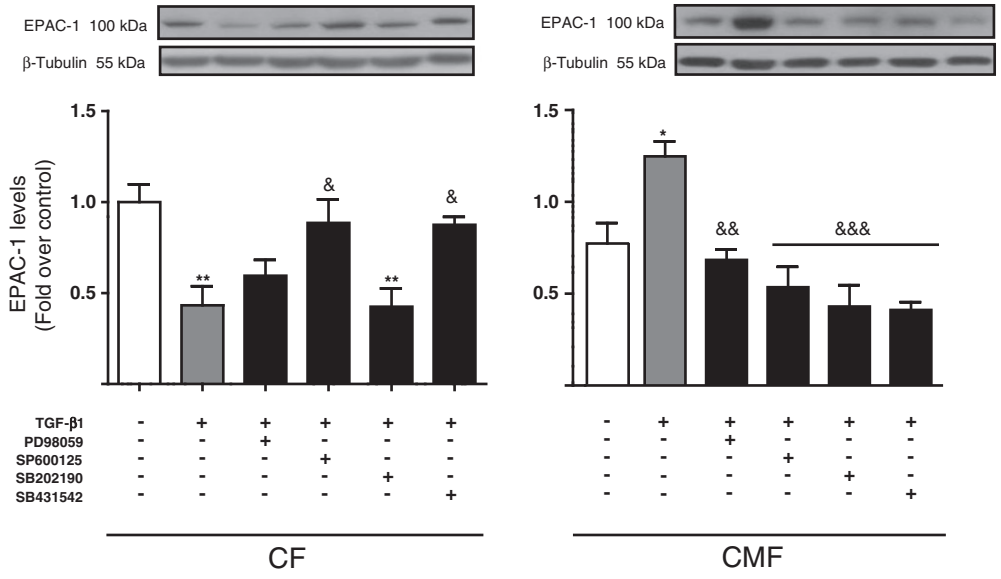

Fig. 2. Signaling pathways activated by TGF-β1 control differentially EPAC-1 expression in cardiac fibroblasts (CF) and myofibroblasts (CMF). TGF-β1 (TGF: 5 ng/mL) decreases EPAC-1 expression in CF (A), while increases EPAC-1 expression in CMF (B), in the presence or absence of PD 98059 (ERK1/2 inhibitor), SB202190 (p38 inhibitor), SP 600125 (JNK inhibitor) and SB431542 (TGF-β1R inhibitor). Protein levels were assessed by western blot; tubulin was used as load control. A representative immunoblot is shown as well as their respective graphic analysis. The results are means (±S.E.M.) of 6 independent experiments. ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs control; $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. TGF-β1).

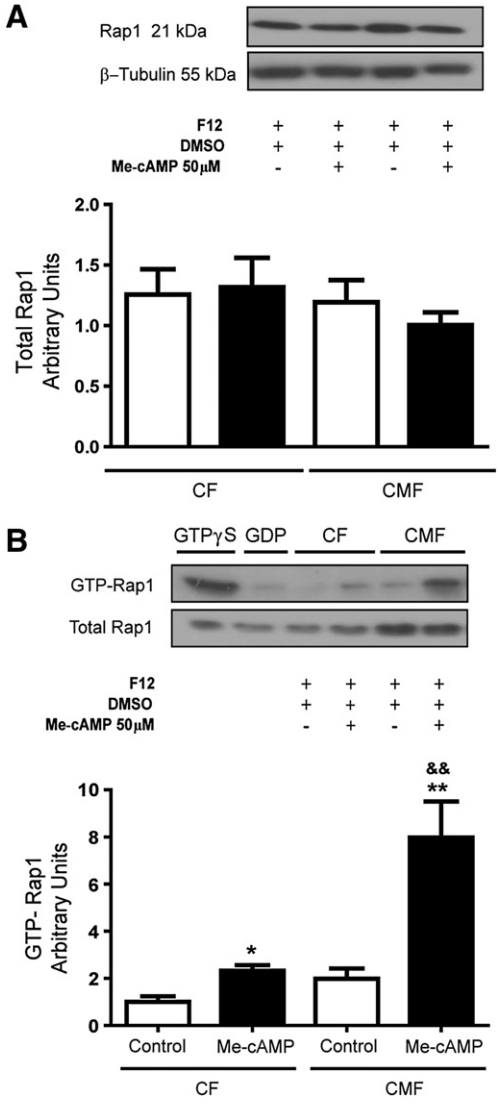

Fig. 3. Cardiac myofibroblasts (CMF) show higher levels of Rap1-GTP than cardiac fibroblasts (CF). (A) Rap1 total expression levels were analyzed in CF and CMF in the presence or absence of Me-cAMP (EPAC selective cAMP analog) by western blot. A representative immunoblot is shown as well as their respective graphic analysis. The results are means (±S.E.M.) of 4 independent experiments. Tubulin was used as load control. (B) Rap1-GTP active levels were analyzed in CF and CMF in the presence or absence of Me-cAMP (EPAC selective cAMP analog), by pull down assay as described in the Methods section. GTPγS and GDP were used as positive and negative controls respectively. Total Rap1 was used as load control. A representative immunoblot is shown as well as their respective graphic analysis. The results are means (±S.E.M.) of 4 independent experiments. (*$p < 0.05$ versus CF control; **$p < 0.01$ vs CMF control and $^{\&\&}p < 0.01$ versus CF Me-cAMP).

with this method were similar to those observed with the scratch method (Fig. 5C).

Both CF and CMF are capable of contracting the ECM in order to initiate and maintain the wound healing process respectively. Fig. 6A shows that both Me-cAMP and isoproterenol induce collagen gel contraction in CF, while no effects were observed with forskolin or the PKA selective cAMP analog. On the other hand, Fig. 6B shows that isoproterenol, Me-cAMP and 6-Bz-cAMP induce contraction of collagen gels in CMF (see Supplementary Figs. 6A–B).

CF and mainly CMF synthesize and secrete ECM components, being fibrillar collagen type I the main component. Figs. 6C and D show that isoproterenol, forskolin, Me-cAMP, and 6-Bz-cAMP reduce the collagen protein levels in both CF and CMF. Angiotensin II was used as a positive control of collagen expression, which significantly increased collagen expression.

Finally, Fig. 7 schematically summarizes the differential regulation of EPAC levels by action of TGF-β1 as well as cellular effects of EPAC in both CF and CMF.

Discussion

The main results of this work showed that TGF-β1 is able to differentially regulate EPAC-1 expression in CF and CMF, and furthermore, we elucidated EPAC cellular function associated with cardiac remodeling in both CF and mainly CMF.

TGF-β1 signaling pathways regulate differentially EPAC-1 expression in cardiac fibroblasts and myofibroblasts

It is well known that TGF-β1 stimulates the differentiation of CF to CMF (Gabbiani, 2003). In our laboratory the characterization of this cellular process has been well established. Catalán et al. showed that differentiation of CF to CMF induced by TGF-β1 is accompanied by parallel decrease in inducible nitric oxide synthase (iNOS), cyclooxygenase 1 (COX-1) and by the induction of kinin receptor subtype 1 (B1R) expression (Catalán et al., 2012). Moreover, there are important differences in susceptibility to death by apoptosis between CF and CMF. Specifically, CF were less resistant than CMF to simvastatin-induced apoptosis. This difference may influence the disappearance or the maintenance of the granulation tissue in myocardial infarction, and therefore the prolonged presence of CMF could mean a defective or appropriate scar formation (Copaja et al., 2011).

Our results showed that CF had lower EPAC-1 and α-SMA expression levels compared to CMF. The high EPAC-1 protein level in CMF suggests that during CF to CMF differentiation process, there is an increase in EPAC-1 protein induced by TGF-β1. This increase in protein content could be necessary for CMF to accomplish their cellular function in the cardiac wound healing, such as collagen secretion, adhesion and contraction of ECM. It is known that TGF-β1 plays an important role in the development of fibrotic processes (Leask, 2007). In addition, this cytokine through Smad proteins induces CF to CMF differentiation and increases the synthesis and deposition of ECM (Li et al., 2008; Liu et al., 2006). Adult rat CF express higher levels of EPAC-1 than EPAC-2 protein. Moreover, TGF-β1 at early times reduces EPAC-1 mRNA and protein expression levels without changes in EPAC-2 mRNA and protein levels (Yokoyama et al., 2008). Our results in neonatal rat CF matched these observations; however, TGF-β1 in CMF significantly increased EPAC-1 protein levels. In addition, using repetitive single and not accumulative doses of TGF-β1 we showed that this cytokine has similar effects on EPAC-1 protein levels of those observed with a single treatment. This opposite response to TGF-β1 between CF and CMF might be a new difference between both cell types. Although CMF are derived from CF, both cellular phenotypes are different at structural and functional levels. In this sense, CMF are cells characterized by secreting increased amounts of ECM proteins in comparison with the CF (Van den Borne et al., 2009), they trigger an important contraction of collagen gel matrix (Lijnen et al., 2003). Also, they have a greater number of kinin B1R (Catalán et al., 2012), angiotensin II type 1 receptor (AT1) and TGF-β1 receptors than CF (Weber and Sun, 2000). In many cell types it has been demonstrated that TGF-β1 is able to activate different kinases in order to control various cellular functions (Derynck and Zhang, 2003). In CF our results showed that TGF-β1 activates early (10 min) the MAPK pathway, and later the canonical pathway (Smad2) (30 min). The rapid phosphorylation of MAPK not only indicates the independence of the Smad pathway activation but also the direct involvement of TGF-β1 receptors on the activation of proteins capable of initiating phosphorylation cascade, such as MAPKKK, also called the TAK1 (TGF-β1 activated kinase-1) (Massagué, 2009; Zhang, 2009). Our results indicated that only in CMF, EPAC-1 was regulated by the participation of both TGF-β1 canonical (Smad2/3) and

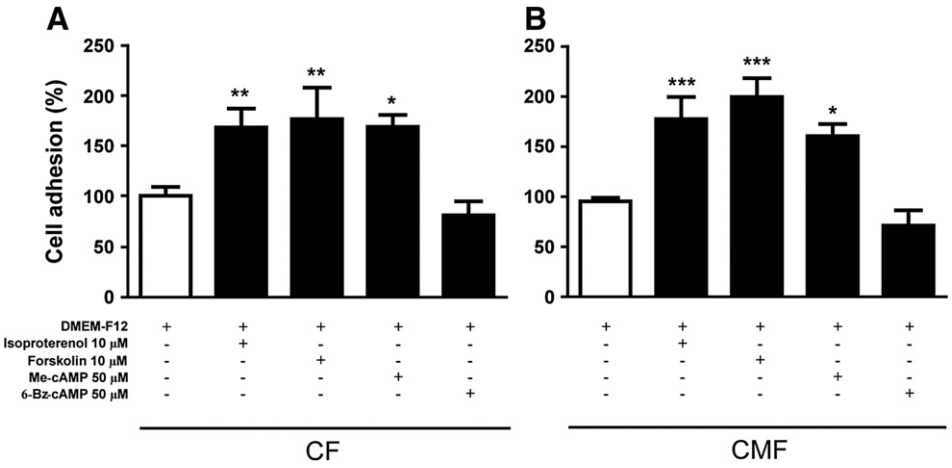

Fig. 4. EPAC regulates cardiac fibroblast (CF) and myofibroblast (CMF) adhesion. (A) CF and (B) CMF were serum starved for 24 h; then the cells were stimulated with isoproterenol (10 μM), forskolin (10 μM), Me-cAMP (50 μM) and 6-Bz-cAMP (50 μM) for 24 h. Cell adhesion assay was performed as described in the Methods section. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control). The results are means (±S.E.M.) of 4 independent experiments.

non-canonical signaling pathways (MAPK and PI3K–Akt). This fact might be the consequence of an increase in expression and/or activity of all proteins involved in TGF-β signaling pathway, which in turn could be a consequence of CF to CMF differentiation process. Data from our laboratory indicates that adult rat CMF have augmented ERK1/2 and Akt phosphorylation basal levels compared to CF. In addition, CMF possess a large amount of TGF-β1 receptor and its ligand, which at the same time is secreted constantly in autocrine manner. This TGF-β1 autocrine stimulation could be able to activate their own receptors and therefore permanently activate their signaling

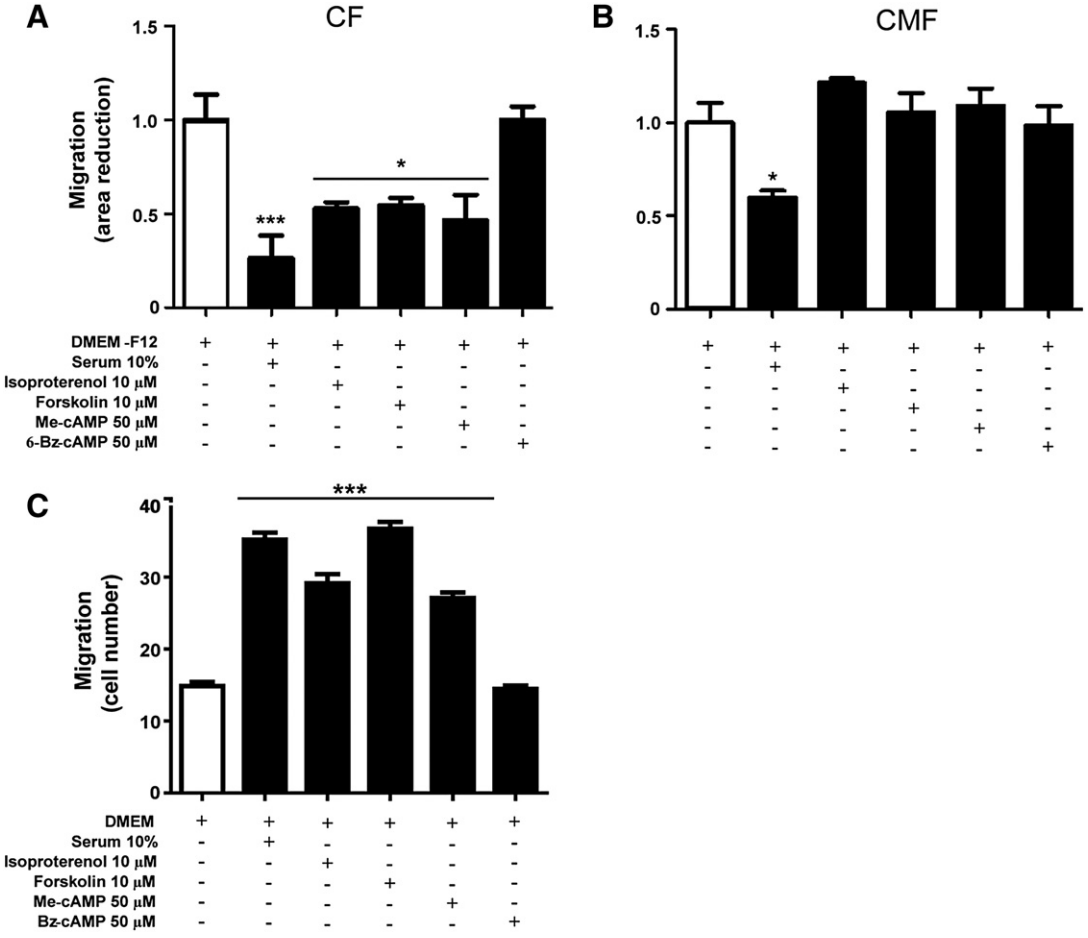

Fig. 5. EPAC regulates cardiac fibroblast (CF) and myofibroblast (CMF) migration. (A) CF and (B) CMF migration was analyzed as described in scratch assay method; (C) CF migration was analyzed as described in Costar Transwell system in the Methods section. The cells were stimulated with isoproterenol (10 μM), forskolin (10 μM), Me-cAMP (50 μM) and 6-Bz-cAMP (50 μM) for 48 h in scratch method and for 2 h in Costar Transwell Assay. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control). The results are means (±S.E.M.) of 4 independent experiments.

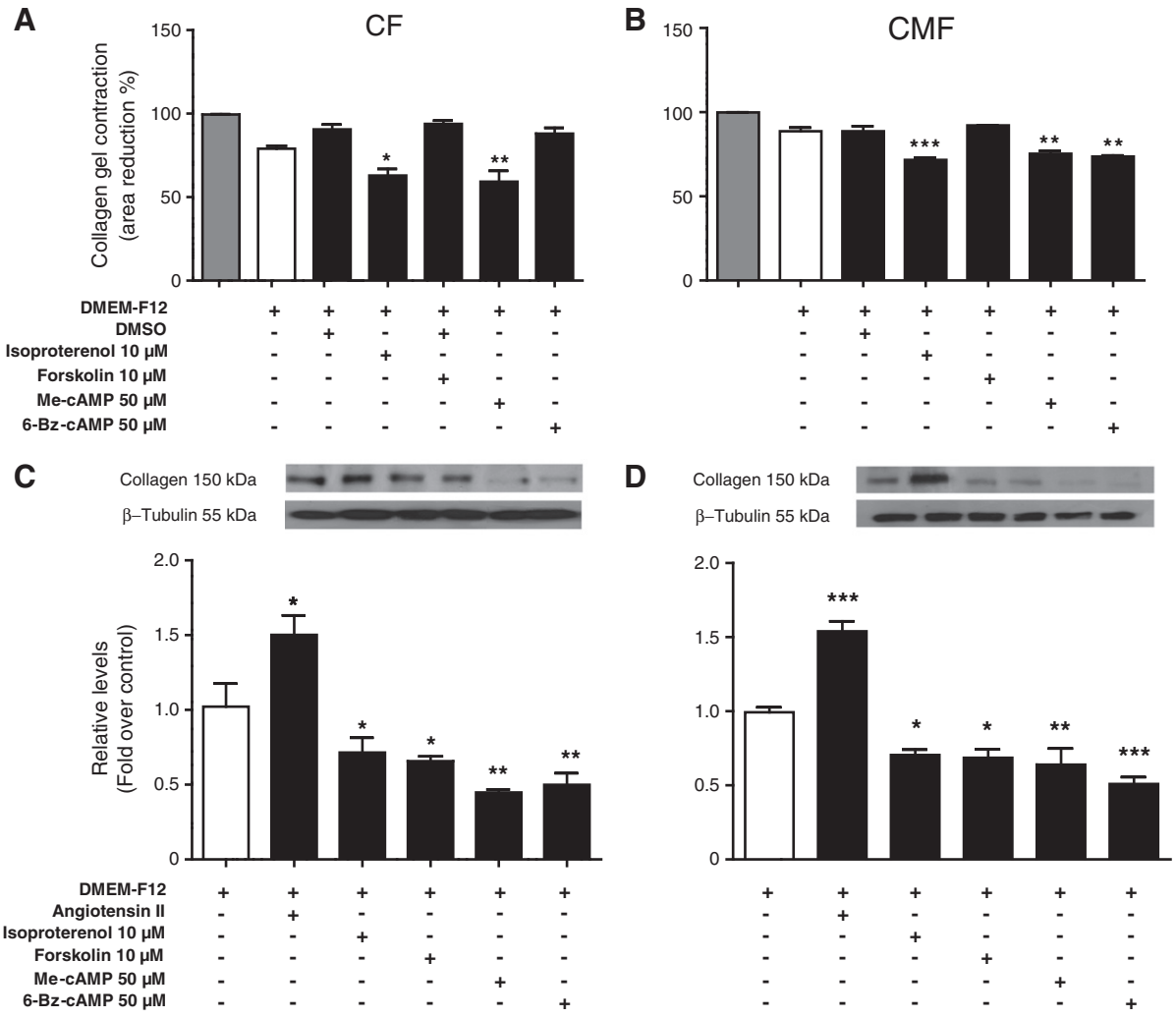

Fig. 6. EPAC regulates differentially collagen gel contraction and collagen synthesis in cardiac fibroblasts (CF) and myofibroblasts (CMF). (A and B) CF and (B) CMF collagen gel contraction was analyzed as described in the Methods section. Cells were stimulated with isoproterenol (10 μM), forskolin (10 μM), Me-cAMP (50 μM) and 6-Bz-cAMP (50 μM). Reduction of collagen area was quantified (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control). The results are means (±S.E.M.) of 4 independent experiments. (C and D) CF and (B) CMF were stimulated with isoproterenol (10 μM), forskolin (10 μM), Me-cAMP (50 μM) and 6-Bz-cAMP (50 μM). Angiotensin II (100 nM) was used as a positive control of collagen expression. Collagen expression levels were analyzed by western blot. A representative immunoblot is shown as well as their respective graphic analysis. The results are means (±S.E.M.) of 6 independent experiments. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control).

pathways. On the other hand, not only the phosphorylation of proteins belonging to TGF-β1 canonical and no canonical signaling pathways is cell-specific, but also the activation of a specific combination of MAPK or Smad by TGF-β1 is dependent on cell type. For example, in rat articular chondrocytes this growth factor is capable of activating ERK1/2 but not JNK and p38 (Javelaud and Mauviel, 2005). Furthermore, in mouse fibroblasts, the JNK signaling pathways have been identified as a key mediator in the response to TGF-β1, and it is necessary for the formation of myofibroblasts and the ECM contraction (Liu et al., 2007). Also, the involvement of MAPK in conjunction with Smads has been shown to be necessary to regulate important regulatory proteins at cardiac level. Recently it was demonstrated that lysyl oxidase, an important extracellular enzyme responsible for the post-translational modification of collagen types I and III, was regulated by TGF-β1 through Smad, PI3K–Akt and MAPK (ERK1/2, JNK and p38) which could work jointly to express this enzyme in adult rat CF (Voloshenyuk et al., 2011). Taken together the results suggest that TGF-β1 participation on EPAC-1 expression is depending on CF or CMF, and thus it is cellular specific. Also the EPAC-1 regulation is depending on canonical and no canonical signaling pathways; apparently in CF less proteins are necessary to control EPAC-1 expression meanwhile all the aforementioned proteins are necessary to control EPAC-1 in CMF. This difference is important because EPAC-1 could be participating in considerable cellular functions associated to wound healing in CF and mainly in CMF.

EPAC function in cardiac fibroblasts and myofibroblasts

EPAC is a guanine nucleotide exchange factor that exerts its function on Rap GTPases Rap1 and 2, small proteins belonging to the superfamily of Ras proteins, which oscillate between an inactive (GDP-bound) and one in active conformation (GTP-bound). Once EPAC is activated by cAMP it is able to stimulate the activity of GDP/GTP exchange on protein Rap, activating and accordingly generating a biological response (Gloerich and Bos, 2010). In this sense our experimental data showed higher levels of Rap1–GTP in CMF compared to CF when an EPAC selective cAMP analog was used as stimulus. There are data in the literature which demonstrate Rap1 activation via EPAC in adult rat CF (Yokoyama et al., 2008), however there is no information regarding with the activation of Rap1 by EPAC in CMF. In this sense the knowledge of this signaling pathway in CMF could be helpful to understand the mechanism by which this

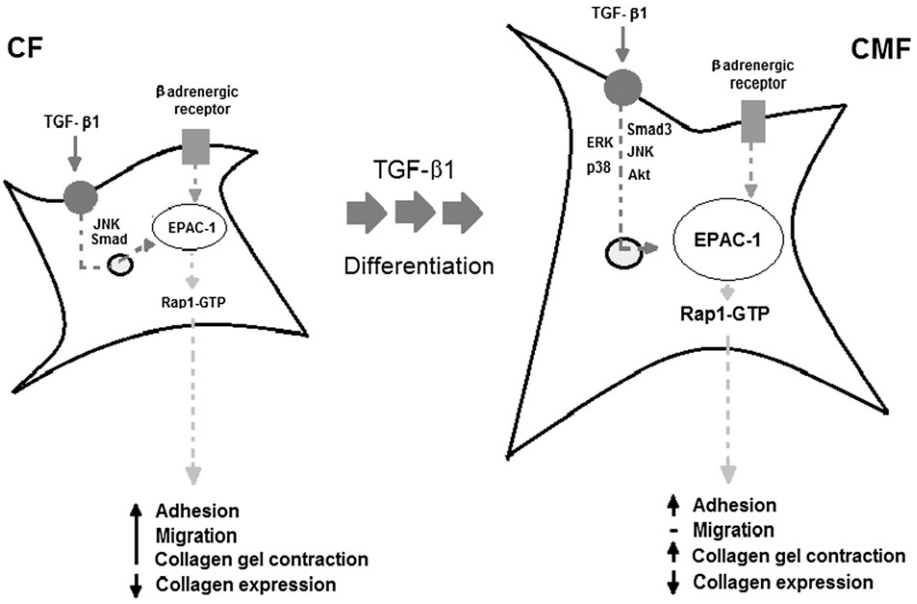

Fig. 7. Schematic representation that summarizes the differential regulation of EPAC levels by action of TGF-β1 both in CF and in CMF; as well as cellular effects regulated by EPAC in both CF and CMF.

phenotype performs the healing process in the heart. To answer this question we performed several experiments in order to evaluate EPAC contribution in processes associated to cardiac remodeling, in which CMF have an important participation. Regarding cell adhesion, isoproterenol and forskolin (both stimuli that increase cAMP synthesis), as well as EPAC selective cAMP analog increased CF and CMF adhesion on fibronectin coated plates, while no effect observed with PKA selective cAMP analog. These results suggest that only EPAC is involved in the adhesion process in both cell types. Our results are consistent with studies in OVCAR3 cells, in which the signaling pathway involving cAMP–EPAC–Rap1 increases the adherence to fibronectin coated plates mediated by integrins (Rangarajan et al., 2003). The study also noted that this process was totally dependent on Rap1 and PKA-independent, which sets a precedent for the involvement of EPAC in cell adhesion processes.

Fibroblast migration plays a fundamental role in tissue healing processes. Our results agreed with those reported in the literature where EPAC is involved in promoting the migration of adult rat CF (Yokoyama et al., 2008). However, CMF did not show migratory capacity under any of the conditions used, which suggests that cAMP/EPAC pathway was not involved in this process. Our results are discordant with respect to migration of myofibroblasts from other tissues or organs. In rat vascular myofibroblasts, TGF-β1 induces expression of potassium channels during the differentiation of fibroblasts to myofibroblasts, through which promotes myofibroblast migration (Liu et al., 2008). Also it has been reported that lipopolysaccharides (LPS) induce migration in human lung myofibroblasts (Li et al., 2012). Although these results are the opposite of those obtained in this work, we must consider that their cellular models are different from ours and that in neither case was EPAC involved. Our data suggest that the cAMP–EPAC/PKA signaling pathway is not involved in migration processes in CMF, but it is possible that this pathway is controlling other functions in this cellular phenotype. In this sense, we must take into consideration that the CMF have large focal adhesion complexes which allow the CMF to be in close and strong contact with ECM (Hinz, 2006). Similarly, we also know that Rap1 is able to activate integrins and strength focal adhesions in epithelial cells (Lyle et al., 2008) and thus inhibit the migration process. A possibility according to our experimental data is probably the EPAC/Rap1 pathway could be strengthening the CMF adhesion contacts with ECM, and thus preventing migration, thereby enhancing the CMF adhesion and contractile activity.

Myofibroblasts express α-SMA protein which confers their contractile property, a very important step during healing process post-myocardial infarction. Both CF and CMF are capable of contracting the ECM in order to perform the wound closure in healing process. Isoproterenol and Me-cAMP induced collagen gel contraction in CF, while forskolin and PKA selective cAMP analog did not affect collagen gel contraction on this phenotype. Moreover, isoproterenol, Me-cAMP and 6-Bz-cAMP induced collagen gel contraction in CMF. These results indicate EPAC participation on collagen gel contraction in CF and CMF; and probably there is an important contribution of Rap1 in this process because, as mentioned before, this GTP-protein is able to strengthen focal adhesions and improve the interaction between cell and the ECM (Lyle et al., 2008). By contrast, in skin fibroblasts, the β2 adrenergic receptor activation delays the collagen gel contraction in a dose dependent manner, suggesting that the stimulus that enables to increase the cAMP content inside the cells has the potential to activate their downstream effector and resist contraction (Pullar and Isserof, 2005). Differences between cardiac and skin cells must be considered in order to explain the distinct response to the same stimulus.

CF and CMF synthesize and secrete ECM components, whose main components are fibrillar collagen types I and III. Under physiological conditions, the ECM preserves cardiac structure and functions primarily through the maintenance of cell shape and for transmitting the contractile forces/mechanical between cardiomyocytes. However, under pathological conditions an excessive deposition of collagen may lead to a disruption of cardiac activity. Our results showed that EPAC and PKA decreased collagen expression. In this sense, other results corroborate our findings. Studies in adult rat CF demonstrated that EPAC activation decreases collagen production induced by angiotensin II in a PI3K-dependent manner, but independent of PKA (Villarreal et al., 2009). However, another study realized on adult rat CF showed that both EPAC and PKA are involved in decrease synthesis of collagen (Yokoyama et al., 2008).

Little is known about how EPAC could be regulating collagen production in CMF, taking into account that this cell phenotype is even more secretory than their precursors the CF. The mechanism by which they could be regulating collagen production is of great

interest. It has been demonstrated that stimulation of kinin B1 receptor leads to a decrease in collagen expression in CMF. The authors of this work proposed that decrease in collagen expression is mainly regulated by cyclooxygenase 2 (COX-2) in CMF (Catalán et al., 2012). In this sense, it is known that COX-2 leads to prostaglandin E2 (PGE2) synthesis, a mediator derived from the metabolism of arachidonic acid by this enzyme; the actions of PGE2 are transduced via activation of the G-coupled E prostanoid 2 receptor which leads to an increase in cAMP (Huang et al., 2008). One explanation to decrease in collagen expression in CMF via EPAC could be through PGE2 synthesis and cAMP production, which in turn will be able to activate EPAC and PKA. However this hypothesis remains to be studied.

In conclusion, the results show that EPAC-1 is regulated differentially by TGF-β1 in CF and CMF and EPAC also modulates the cellular process involved in the remodeling of ECM, strengthening the concept of profibrotic (CF adhesion and migration) and the antifibrotic effects (decreased collagen synthesis in CF and CMF). These results show EPAC as a pharmacological target for selectively controlling processes associated with tissue healing.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.06.022.

Conflict of interest

None declared.

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