Toxicology and Applied Pharmacology 261 (2012) 300–308

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Differential regulation of collagen secretion by kinin receptors in cardiac fibroblast and myofibroblast

Mabel Catalán [a], Christian Smolic [a], Ariel Contreras [b], Pedro Ayala [a], Ivonne Olmedo [a], Miguel Copaja [a], Pía Boza [a], Raúl Vivar [a], Yennifer Avalos [a], Sergio Lavandero [a,b,d], Victoria Velarde [c], Guillermo Díaz-Araya [a,*]

[a] Centro de estudios moleculares de la célula, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Chile
[b] Instituto Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Chile
[c] Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile
[d] Department of Internal Medicine (Cardiology Division), University of Texas Southwestern Medical Center, Dallas, TX, USA

ARTICLE INFO

Article history:
Received 16 January 2012
Revised 28 March 2012
Accepted 9 April 2012
Available online 18 April 2012

Keywords:
Kinin receptor
Fibroblast
Myofibroblast
Collagen
Heart

ABSTRACT

Kinins mediate their cellular effects through B1 (B1R) and B2 (B2R) receptors, and the activation of B2R reduces collagen synthesis in cardiac fibroblasts (CF). However, the question of whether B1R and/or B2R have a role in cardiac myofibroblasts remains unanswered.

Methods: CF were isolated from neonate rats and myofibroblasts were generated by an 84 h treatment with TGF-β1 (CMF). B1R was evaluated by western blot, immunocytochemistry and radioligand assay; B2R, inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and cyclooxygenases 1and 2 (COX-1, and COX-2) were evaluated by western blot; intracellular Ca^{+2} levels were evaluated with Fluo-4AM; collagen secretion was measured in the culture media using the picrosirius red assay kit.

Results: B2R, iNOS, COX-1 and low levels of B1R but not eNOS, were detected by western blot in CF. Also, B1R, B2R, and COX-2 but not iNOS, eNOS or COX-1, were detected by western blot in CMF. By immunocytochemistry, our results showed lower intracellular B1R levels in CF and higher B1R levels in CMF, mainly localized on the cell membrane. Additionally, we found B1R only in CMF cellular membrane through radioligand displacement assay. Bradykinin (BK) B2R agonist increased intracellular Ca^{2+} levels and reduced collagen secretion both in CF and CMF. These effects were blocked by HOE-140, and inhibited by L-NAME, 1400W and indomethacin. Des-Arg-kallidin (DAKD) B1R agonist did not increase intracellular Ca^{2+} levels in CF; however, after preincubation for 1 h with DAKD and re-stimulation with the same agonist, we found a low increase in intracellular Ca^{2+} levels. Finally, DAKD increased intracellular Ca^{2+} levels and decreased collagen secretion in CMF, being this effect blocked by the B1R antagonist des-Arg9-Leu8-kallidin and indomethacin, but not by L-NAME or 1400 W.

Conclusion: B1R, B2R, iNOS and COX-1 were expressed differently between CF and CMF, and collagen secretion was regulated differentially by kinin receptor agonists in cultured CF and CMF.

Introduction

Cardiac fibroblasts (CF) are the major non-myocyte cell constituent in the myocardium and they are involved in cardiac remodeling. Notably, transforming growth factor beta 1 (TGF-β1) stimulates the fibroblast to differentiate into myofibroblast (Gabbiani, 2003). Differentiated CMF play a central role in cardiac matrix remodeling, as they contribute to abnormal expansion of the interstitium and, ultimately, to cardiac fibrosis. Compared with relatively quiescent CF, CMF are hypersecretory for fibrillar collagens, and these collagen subtypes provide tensile strength for the healing of wounds and scar stabilization, thus preventing rupture (Porter and Turner, 2009; Baum and Duffy, 2011).

Kinins are known to control relevant cardiovascular effects, such as vascular dilation and permeability, myocardial glucose uptake, matrix regulation and myocardial growth (Moreau et al., 2005). Moreover, kinins play an important role in the pathophysiological processes that accompany inflammation, as well as tissue damage and repair (Maurer et al., 2011). Bradykinin (BK) and Lys-BK, or kallidin (KD), are formed from kininogen precursors and are rapidly metabolized by proteolytic enzymes to form active fragments, such as des-Arg9-BK (DABK) and des-Arg10-KD (DAKD). Kinin actions are mediated through the receptor subtypes B1 (B1R) and B2 (B2R) (Moreau et al., 2005). The B2R is constitutively expressed in a diverse range of tissues and mediates the action of BK and KD. Evidence from animal models

Abbreviations: B1R, bradykinin 1 receptor; B2R, bradykinin 2 receptor; BK, bradykinin; CF, cardiac fibroblasts; CMF, TGF-beta-treated CF; COX, cyclooxygenase; DAKD, des-Arg10-KD; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase, 1400W inducible nitric oxide synthase inhibitor; IL-1β, interleukin-1β; KD, kallidin or Lys-BK; L-NAME, L-NG-Nitroarginine methyl ester; leu8, des-Arg9-Leu8-kallidin; NO, nitric oxide; PG, prostaglandins; PLA2, phospholipase A2; α-SMA, α-smooth muscle actin; INDO, indomethacin; WB, western blot; ICC, immunocytochemistry.

* Corresponding author at: Facultad Ciencias Químicas y Farmacéuticas, Universidad de Chile, Sergio Livingstone 1007, Santiago 8380492, Chile. Fax: +56 2 978 2912.
E-mail address: gadiaz@ciq.uchile.cl (G. Díaz-Araya).

0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2012.04.013

has suggested that B2R activation is responsible for most of the actions of kinins under normal conditions. In contrast, the B1R is rapidly induced *in vivo* under stress conditions and mediates the action of DABK and DAKD (Leeb-Lundberg et al., 2005). B1R is expressed in lung fibroblasts and alveolar macrophages after exposure to noxious stimuli, including the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Calixto et al., 2004; Phagoo et al., 2001). Additionally, kinin receptors activate several second messenger systems, depending on cell type (Moreau et al., 2005). Signaling transduction pathways have been comprehensively studied in endothelial cells. They are indirectly linked to cyclic nucleotide signaling, via the production of nitric oxide (NO), which activates the soluble guanylate cyclase in neighboring muscle cells. Furthermore, kinins can induce arachidonic acid liberation into the intracellular space and stimulate cyclooxygenases (COX-1 and COX-2), so as to produce prostaglandins (PG) I2 or E2 that can interact with their own receptors (Moreau et al., 2005). The activation of ionic channels and phospholipases A2, C, and D also plays a role in kinin receptor signaling. Thus, phospholipase C products, inositol 1,4,5-triphosphate and diacylglycerol (Moreau et al., 2005) are responsible for the increase in intracellular Ca^{2+} and for protein kinase C activation, respectively. In addition, Ca^{2+} can participate in the activation of endothelial NO synthase (eNOS), and ultimately in NO production and stimulation of phospholipase A2 (PLA2) (Leeb-Lundberg et al., 2005). Although the B1R and B2R have similar intracellular transduction pathways, the signaling patterns are different in terms of Ca^{2+} concentration variation, as well as regarding the susceptibility to desensitization of both receptors (Mathis et al., 1996). However, these signaling pathways have not been studied in depth in CF, and even less in CMF.

CF constitutively express the B2R and kinins have shown to reduce collagen secretion (Kim et al., 1999). However, it is unknown whether the B1R or B2R are expressed in CMF and how collagen synthesis is regulated by kinins in CF and CMF. The aims of this study were as follows: a) to evaluate the presence of B1R and B2R in CF and CMF; and b) to study the molecular effectors involved in the reduction of collagen secretion by kinins.

Materials and methods

Isolation and culture of CF and CMF

Rats were obtained from the Animal Breeding Facility of the School of Chemical and Pharmaceutical Sciences at the Universidad de Chile. All studies followed 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. CF were prepared from hearts of 1–3 day-old Sprague–Dawley rats, as previously described (Diaz-Araya et al., 2003). TGF-β1 treated CF (CMF) that resemble cardiac myofibroblasts obtained from infarcted hearts, were prepared from neonatal rat CF as follows: CF (at the second passage, $20\times10^3/cm^2$) were plated on plastic dishes, serum deprived and treated with TGF-β1 (5 ng/mL) for 84 h in Dulbecco's minimal essential medium (DMEM)-F12. CF to CMF differentiation was analyzed by immunocytochemistry, using antibodies against vimentin and α-smooth muscle actin (α-SMA).

Cells were treated with or without the B1R agonist DAKD (100 nM) or the B2R agonist bradykinin (100 nM). The B1R antagonist Des-Arg9-Leu8-kallidin (leu8; 10 μM), the B2R antagonist HOE-140 (10 μM), the general NOS inhibitor L-NAME (10 μM), the specific iNOS inhibitor 1400W (10 μM), or the COX-1 and COX-2 inhibitor indomethacin (10 μM) were added to cultures 1 h before treatment with DAKD or BK.

Western blot (WB)

Cell proteins were extracted with a protease inhibitor cocktail-containing lysis buffer. Aliquots were resolved on 12% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with primary antibodies against vimentin (1:1000) and α-SMA (1:5000) from Sigma-Aldrich (St Louis, MO). B1R (1:1000), B2R (1:1000), iNOS (1:1000), eNOS (1:1000), COX-1 (1:1000), COX-2 (1:1000) and tubulin (1:1000) were obtained from Santa Cruz Biotech (Santa Cruz, CA). All of them were incubated at 4 °C overnight. Bound antibodies were detected by horseradish peroxidase conjugated secondary antibody and visualized by ECL reagent.

Measurements of intracellular Ca^{2+} levels ($[Ca^{2+}]i$)

Intracellular Ca^{2+} levels were determined in CF and CMF preloaded with FLUO-4AM (50 μM, 30 min) by using an inverted confocal microscope (Carl Zeiss Axiovert 135 M-LSM Microsystems).

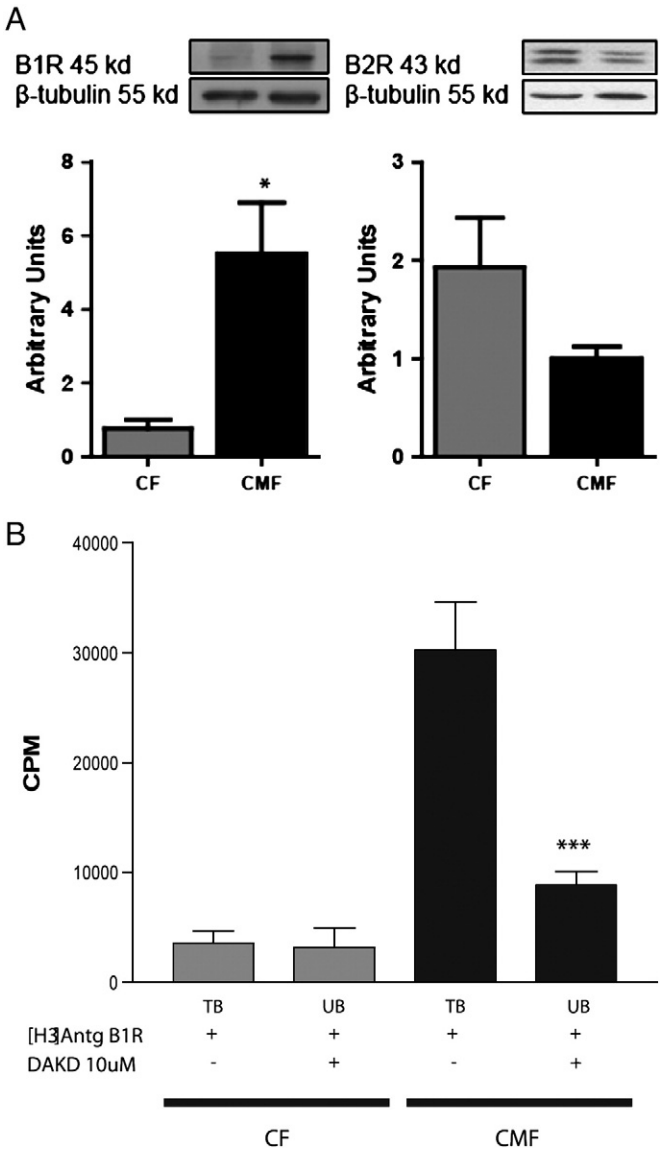

Fig. 1. Expression of B1R and B2R in cardiac fibroblast (CF) and myofibroblast (CMF), and B1R radioligand displacement assay in CF and CMF. (A) Representative western blots demonstrating that B1R and B2R are present in CF and CMF, and the graph analysis is also shown. Results are exposed as means (±S.E.M.) of 3 separate experiments. *$p<0.05$ for CMF v/s CF. **(B)** [3H][Leu9]des-Arg10-[3,4-propil-3,4H(N)]kallidine ([3H]-Antg B1R), as radioligand for displacement assays in CF and CMF with DAKD (10 μM). The assays were done as described in the Materials and methods section herein. Results shown are means (±S.D.) of three separate experiments. ***$p<0.001$ in CMF for TB v/s UB (TB: total binding; UB: unspecific binding).

Radioligand binding assay

We used the specific B1R antagonist [3H][Leu9]des-Arg10-[3,4-propil-3,4H(N)]kallidine ([3H]-Antg B1R) for binding assays that were performed on CF and CMF membranes, as previously described (Aránguiz-Urroz et al., 2009). For competitive binding experiments, [3H]-Antg B1R (1 nM) was used as a labeled compound. Non-labeled DAKD agonist (10 μM) was used to confirm the kinin receptor subtype.

Immunocytochemistry (ICC)

Cell cultures were grown close to 70% of confluence on glass coverslips in complete medium containing 10% fetal calf serum (FBS). Then, cells were serum-deprived for 24 h, and treated with DAKD (for 30 min) or TGF-β1 (for 96 h). TGF-β1 induced morphological changes were monitored by phase-contrast microscopy. After treatment, cells were fixed at room temperature for 10 min in 3.7% formaldehyde (v/v) in phosphate-buffered saline (PBS). They were then washed and blocked with serum-containing buffer (10% FBS in PBS with 0.02% sodium azide), and cells were subsequently incubated with antibody anti vinculin, anti α-SMA, and anti-B1R for 2 h at room temperature. The secondary antibody that was used was conjugated with FITC. Coverslips were mounted on glass slides with Dako mounting medium, and micrographs were obtained with a confocal microscope (Carl Zeiss, Germany).

Collagen secretion of cultured CF and CMF

Total soluble collagen in cell culture supernatants was quantified using the SirCol collagen assay (Biocolor, Belfast, Ireland), following the protocol described by the manufacturer.

Statistical analysis

Data are mean (±SEM) of at least 3 independent experiments. *t*-Student test for comparisons between 2 groups or one-way ANOVA followed by a Tukey's *post hoc* test for multigroup comparisons was used. Significance was set at $p<0.05$.

Results

Expression of B1 and B2 receptors in CF and CMF

Supplementary Fig. 1, shows by ICC that cultured CF and CMF were vimentin positive. However, only CMF showed prominent and well developed α-SMA fibers. In addition, by WB we observed that CMF express α-SMA; although low levels were found in CF they are not forming fibers. B2R WB analysis of cell protein extracts from CF and CMF showed a band with an apparent molecular mass of 43 kDa, with higher expression in CF than in CMF (Fig. 1A, right panel). In contrast, WB analysis showed a band with an apparent molecular mass of 45 kDa for B1R, having a higher expression in CMF than in CF (Fig. 1A, left panel). Both molecular masses are close to those calculated for human B2R and B1R, respectively. β-tubulin was used as loading control.

Radioligand binding competition assay

Radioligand binding was performed with membrane preparations. Data from saturation analysis obtained with B1R selective agonist radioligands, [3H]-Antg B1R (1 nM), showed specific displacement from CMF, but not from CF (Fig. 1B). The reference agonist DAKD

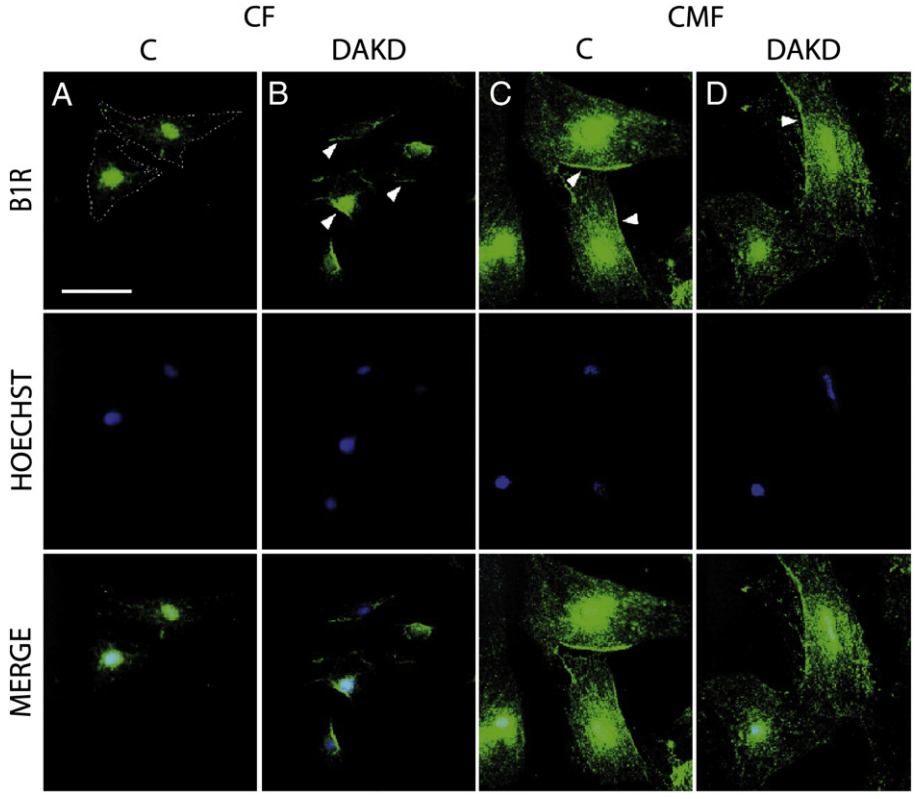

Fig. 2. Immunocytochemistry analysis of B1R in cardiac fibroblasts (CF) and myofibroblast (CMF). CF (A and B) or CMF (C and D) were treated with vehicle (A and C) or with DAKD (100 nM) (B and D) for 30 min, and then the cells were fixed, permeabilized, and immunostained with antibody to B1R, and visualized by fluorescence microscopy. Low levels of B1R were localized in CF, mostly in perinuclear vesicles (A), while a higher B1R positive immunostain was localized in CMF, mostly on cell membrane (C) (white arrow), and in perinuclear vesicles. In DAKD pretreated CF, higher immunostain was localized on cell membrane (B) (white arrow), while no difference was found in DAKD pretreated CMF (D). The results are shown as representative of 3 independent experiments; scale bar, 50 μm).

was very potent in displacing the radioligand only in CMF cell membranes.

Immunolocalization of B1R on CF and CMF

Fig. 2 shows the levels and localization of B1R on CF and CMF by ICC. In normal CF, immunoreactive B1R was found mostly in intracellular vesicles. However, in CF pretreated with DAKD we found positive immunoreactive B1R in intracellular vesicles, and also on the cellular membrane (white head arrows). In CMF we observed higher B1R immunostaining, localized mostly on the cell membrane. Nevertheless, we did not find any difference with control in DAKD pretreated CMF.

Effects of the B2R agonist BK and the B1R agonist DAKD on $[Ca^{2+}]_i$ in CF and CMF

To investigate whether B2R is functional in CF, and if B1R and B2R are functional in CMF, we tested the effect of their specific agonists on intracellular calcium levels ($[Ca^{2+}]_i$). When BK was added as a ligand, B2R was activated and a transient raise of $[Ca^{2+}]_i$ was observed in CF (Fig. 3A) as well as in CMF (Fig. 3B). Using HOE-140, a specific B2R antagonist, we showed that such increase in $[Ca^{2+}]_i$ stimulated by BK was blocked in both cell types (Figs. 3C and D). However, Leu8, the specific B1R antagonist, did not block the increment of $[Ca^{2+}]_i$ in CF or in CMF stimulated with BK (Figs. 3E and F). These results confirmed that BK selectively activated the B2R, both in CF and CMF. Additionally, Fig. 4A shows that B1R activation by DAKD did not raise $[Ca^{2+}]_i$ in CF. Conversely, a robust but transient increase in $[Ca^{2+}]_i$ was observed in CMF (Fig. 4C), in which this effect was blocked by Leu8 (Fig. 4D), but not by HOE-140 (Fig. 4E). This confirms that B1R is functional in CMF. Finally, we observed a small increment of $[Ca^{2+}]_i$ when CF pretreated for 1 h with DAKD, were stimulated with DAKD for a second time (Fig. 4B). This last result and the WB analysis confirm that B1R is present in CF, although it is not active in non-DAKD pretreated CF.

eNOS, iNOS, COX-1 and COX-2 protein levels in CF and CMF

eNOS immunoreactive bands were not detected in cultured CF and CMF, although an immunoreactive band was found in endothelial cells used as positive control (PC) (140 kDa) (Fig. 5A). WB probed with anti-iNOS and anti-COX-1 antibodies demonstrated immunoreactive bands only in CF. The molecular mass of the immunoreactive band for iNOS and COX-1 found in CF was 130 kDa and 70 KDa, respectively, and were consistent with the previously described molecular masses for these proteins (Figs. 5A and B). COX-2 was detected both in CF and CMF; the molecular mass of the immunoreactive band found in both cells was 72 kDa and was consistent with the previously described molecular mass for COX-2 (Fig. 5B).

Effects of the B1R and B2R agonists on collagen secretion in CF and CMF

We tested the effects of BK and DAKD on collagen protein secretion in CF and CMF by analyzing the supernatants of BK and DAKD-treated CF and CMF, respectively (Figs. 6A and B). Collagen secretion levels were higher in CMF than in CF, and BK strongly decreased collagen secretion in both cell types when compared to control (Fig. 6A). DAKD also reduced collagen secretion, but only in CMF (Fig. 6B).

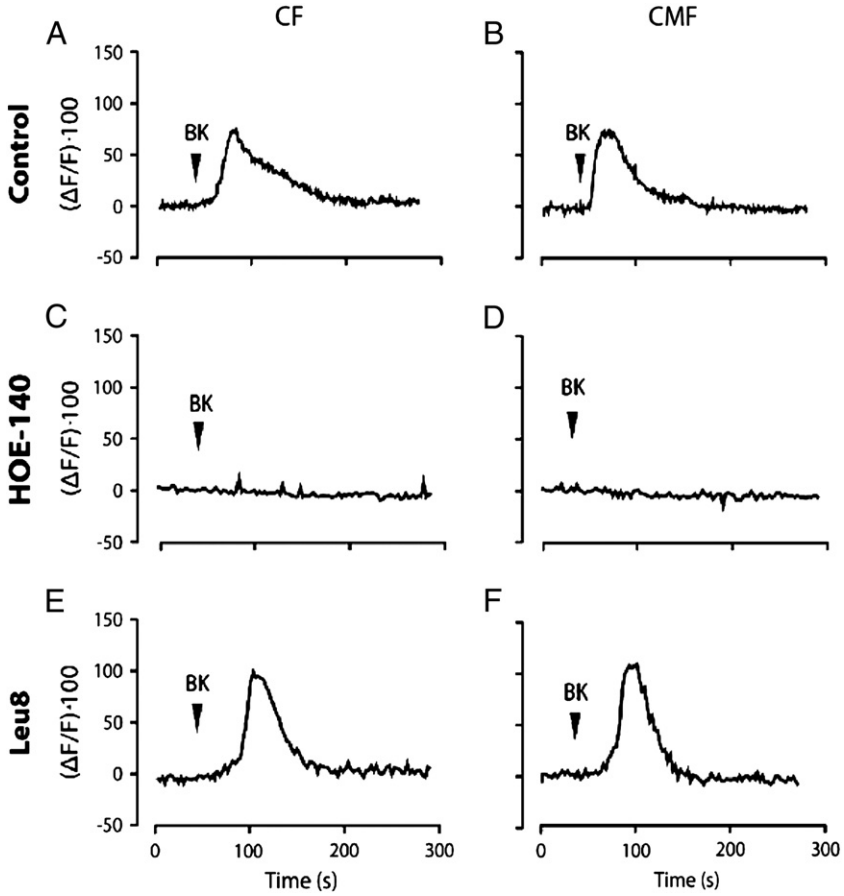

Fig. 3. BK triggers intracellular calcium increase by B2R in cardiac fibroblast (CF) and myofibroblast (CMF). CF and CMF were loaded with Fluo-4/AM and treated with B2R agonist (bradykinin: BK 100 nM). Typical profiles on intracellular Ca^{2+} levels in response to BK are observed in CF (A) and CMF (B), in the presence of HOE-140 as B2R antagonist (C and D), or in the presence of Leu8 as B1R antagonist (E and F). The fluorescence ratios are representative data for 3 similar and independent experiments.

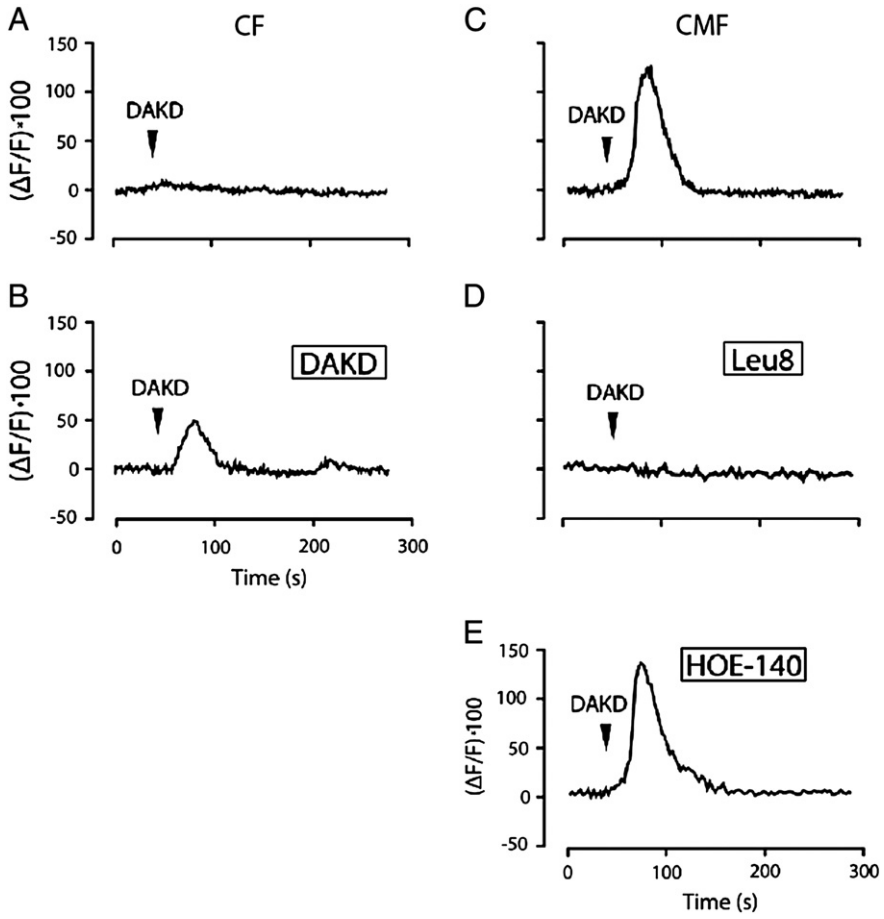

Fig. 4. DAKD triggers intracellular calcium increase by B1R in cardiac fibroblasts (CF) and myofibroblasts (CMF). CF and CMF were loaded with Fluo-4/AM and treated with B1R agonist (DAKD, 100 nM). Typical profiles of intracellular Ca^{2+} levels in response to DAKD are observed in CF (A) and CMF (C), in the presence of Leu8 as B1R antagonist (D), or in presence of HOE-140 as B2R antagonist (E). A low increase in Ca^{2+} levels was observed in CF pretreated with DAKD (100 nM) for 30 min prior to a second stimulus with DAKD (100 nM) (B). The fluorescence ratios are representative data of 3 similar and independent experiments.

Modulation of B2R and B1R signaling pathways involved in collagen secretion in CF and CMF

We tested the effects of the B2R antagonist, NOS and COX inhibitors on BK-reduced collagen secretion in CF and CMF (Figs. 6C and D, respectively). Collagen secretion was not reduced in cells preincubated with HOE-140 and stimulated with BK, thus maintaining similar levels to unstimulated CF and CMF. Similarly, when cells were preincubated with L-NAME or 1400 W and stimulated with BK, collagen secretion did not change compared to control in CF. However, BK decreased collagen secretion even in the presence of L-NAME or 1400W in CMF. Finally, in cells pretreated with indomethacin, BK did not reduce collagen secretion in both cell types. HOE-140 or the inhibitors themselves did not exhibit any effect on basal secreted collagen. These results suggest that although BK decreases collagen secretion in CF and CMF through the B2R, the signaling pathways activated in both cell types are different, involving iNOS and COXs in CF, and only COX-2 in CMF.

Accordingly, we tested the effects of B1R and B2R antagonists, NOS and COXs inhibitors on the reduction of collagen secretion induced by DAKD in CMF. DAKD caused collagen secretion to significantly decrease in a concentration dependent manner, being maximal at 100 nM (Fig. 6E). When the B1R was blocked by Leu8, DAKD did not reduce collagen secretion, as compared to levels observed in non-stimulated CMF. The addition of DAKD in the presence of HOE-140 reduced collagen secretion, which was similar to what was found with DAKD alone. DAKD decreased collagen secretion even in the presence of L-NAME or 1400W (Fig. 6F). Nevertheless, indomethacin prevented this reduction. The antagonist or the inhibitors themselves did not exhibit any effect on basal secreted collagen. Taking into account our previous observations, these results indicate that DAKD decreases collagen secretion in CMF through a pathway mediated by B1R and COX-2.

Discussion

It is well established that TGF-β1 stimulates the differentiation of CF to CMF (Gabbiani, 2003). Differentiation to a myofibroblast phenotype can contribute to hypersecretion of ECM proteins, and these hypersecretory cells are important players in the wound healing and cardiac remodeling processes (Van den Borne et al., 2010). The amount of CMF diminishes via apoptosis during scar maturation in the heart (Hayakawa et al., 2003). Thus, a lack of adequate myofibroblasts apoptosis could lead to ECM protein overproduction. Therefore, the regulation of collagen secretion is highly relevant in this context.

Using our experimental model, we showed that the differentiation from CF to CMF is accompanied by increased B1R expression and membrane localization. Our western blot results indicated the presence of B1R in CF, while immunolocalization showed that the B1R is mainly found in intracellular vesicles, and lower immunolocalization was observed on cell membrane. Also, our results did not show radioligand displacement, indicating that the B1R is not present in membrane fraction. The latter assertion is strengthened by immunolocalization results showing that pretreatment with the DAKD mobilizes B1R towards the cellular membrane. Similar results have been observed in HeLa cells, where pretreatment with the B1R agonist was shown to trigger

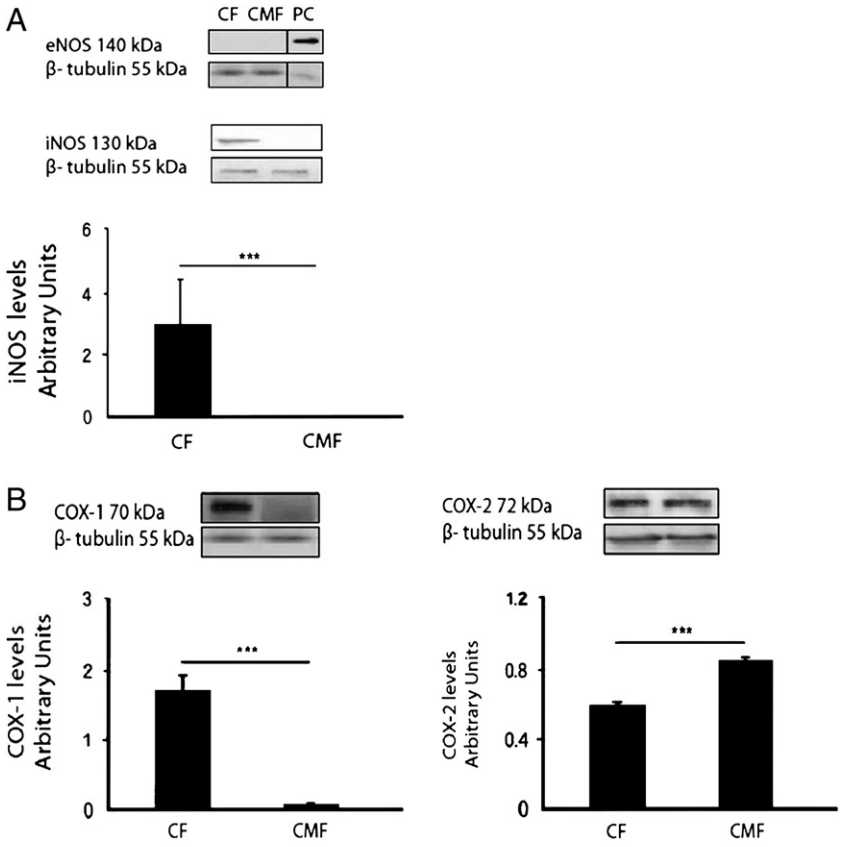

Fig. 5. Expression levels of iNOS, eNOS, COX-1 and COX-2 in cardiac fibroblasts (CF) and myofibroblasts (CMF). (A) Representative western blots demonstrating that iNOS is present only in CF, while eNOS is not present in either cells; the graphic analysis for iNOS level is shown. (B) Representative western blots demonstrating that COX-1 is present only in CF, while COX-2 is present in both cells (p.c.: positive control are endothelial cells); the graph analysis is also shown. The results exposed are means (±S.E.M.) of 3 separate experiments. ***$p<0.05$ CMF v/s CF. Tubulin was used as load control.

the displacement of this receptor from intracellular vesicles to the cell membrane (Enquist et al., 2007).

To our knowledge, this is the first report showing a dynamic change in the expression levels of B1R in CF, and although the stimulus responsible for this effect was not investigated here, TGF-β1 is an obvious candidate. In this respect, both kinin receptors and TGF-β1 have been shown to be up-regulated in myocardial ischemia. However, IL-1β could also be implicated. Phagoo et al. (2001) reported that this cytokine can activate components of the kallikrein–kinin system. Moreover, it has been established that IL-1β is capable of producing the up-regulation of B1R and B2R in adult CF (Imai et al., 2005). Nevertheless, other authors have shown that IL-1β is incapable of inducing any kinin receptor in neonatal CF (Riad et al., 2008). Interestingly, induction and release of proinflammatory IL-1β is also consistently reported in experimental models and patients with myocardial infarction (Frangogiannis, 2007; Balbay et al., 2001). In addition, TGF-β1 induces the secretion of IL-1β in synovial fibroblast (Cheon et al., 2002). However, both possibilities should be clarified in further studies. Our data clearly shows that B1R is more abundant in CMF and, therefore, this receptor may become the dominant subtype in mediating chronic heart inflammation.

In this study, the functionality of B2R and B1R in CF and CMF was assessed by evaluating the kinin receptor-mediated $[Ca^{2+}]_i$ mobilization in single cells. These results depicted similar patterns of $[Ca^{2+}]_i$ signals elicited by the stimulation of kinin receptors in cultured CF and CMF. Our data shows that B2R was functional both in CF and CMF, while the B1R was only functional in CMF. In this sense, our results indicate that DAKD did not induce an increment of $[Ca^{2+}]_i$ in CF, but a new stimulus with the same agonist was able to induce greater $[Ca^{2+}]_i$ in CF pretreated with DAKD for 1 h. These results collectively strengthen our previous findings using WB, radioligand assay and ICC for B1R expression levels and localization, and are in agreement with the results observed in HeLa cells (Enquist et al., 2007). It is described in the literature that pretreatment with DAKD is able to mobilize B1R from its location in the intracellular vesicles to the cell membrane, by a mechanism that involves the presence of a small amount of the B1R on the cell membrane. In this sense, Enquist et al. (2007) demonstrated that DAKD pretreatment for 30 min increased the number of radioligand binding sites. These last results strengthen our findings and suggest that B1R is localized within intracellular vesicles in CF. Finally, from our results we cannot conclude whether this increase in $[Ca^{2+}]_i$ levels induced by the stimulation of B1R or B2R is derived from intracellular stores or by external influx. However, it has been established that BK induces intracellular calcium release in human CMF (Riches et al., 2010).

BK receptor signaling pathways involve the activation of several second messenger systems (Moreau et al., 2005), and some of them have been implicated for collagen secretion regulation (Kim et al., 1999; Gallagher et al., 1998). We identified COXs and NOS as probable molecular effectors along these signaling pathways in CF and CMF. Interestingly, a differential expression of these enzymes in the two cell types was observed. CF did not express eNOS, but expressed iNOS, COX-1 and COX-2, whereas only this last cyclooxygenase was detected in CMF and at higher levels than that of CF. NO generation has been shown to be involved in collagen secretion (Kim et al., 1999) and iNOS could be the main isoform implicated for NO synthesis in CF. Additionally, a reduction of iNOS levels induced by TGF-β1 was reported in an ischemic heart model (Pinsky et al., 1995). Whether BK or DAKD increase NO in CF and CMF was not evaluated here, but some evidence shows that BK induces NO generation in CF (Kim et al., 1999). This suggests that NO could mediate the effects of BK or DAKD in our cells.

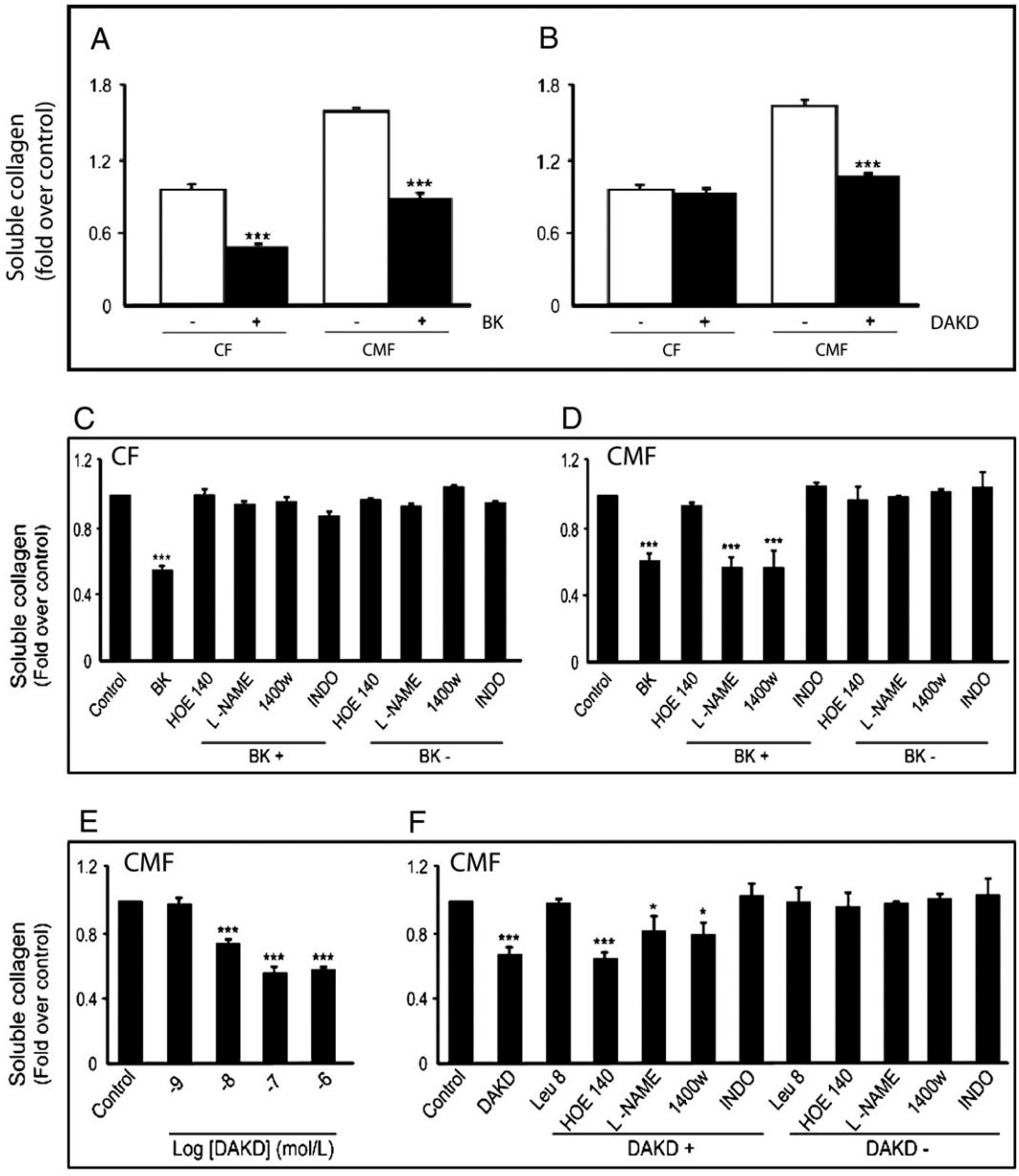

Fig. 6. Decrease in soluble collagen secretion by BK or DAKD in cardiac fibroblasts (CF) and myofibroblasts (CMF). (A) Bradykinin (100 nM BK for 48 h) decreases soluble collagen secretion in CF and CMF. (B) Des-Arg-kallidin (100 nM DAKD for 48 h) decreases soluble collagen secretion in CMF, but not in CF. The drop in soluble collagen secretion in CF (C) or CMF (D) preincubated with HOE-140 (10 μM), 1400W (10 μM), L-NAME (10 μM) or indomethacin (10 μM) with/without bradykinin (BK 100 nM; for 48 h), was inhibited by L-NAME or 1400W only in CF (C). (E) Typical concentration-dependent effects of DAKD for 48 h (1 nM to 10 μM) on soluble collagen secretion in CMF. (F) The decrease of soluble collagen secretion in CMF by DAKD was blocked by preincubating the cells with leu8 (10 μM) and indomethacin (10 μM), but not with HOE-140 (10 μM), 1400W (10 μM) and L-NAME (10 μM). Results shown are means (±S.E.M.) of 3 separate experiments. The assays were done as described in the Materials and methods section. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ v/s control.

COXs are rate-limiting enzymes that catalyze the first step in prostanoid synthesis (prostaglandin and thromboxane), and their involvement in collagen secretion has also been observed (Gallagher et al., 1998; Yu et al., 1997). COXs are present in at least two isoforms, namely COX-1 and COX-2, which catalyze identical reactions. COX-1 is constitutively expressed in many cells, where it produces prostanoids for normal physiological functions. During the differentiation of CF to CMF induced by TGF-β1, we observed a significant down-regulation of COX-1. This is the first report showing this effect and there are no other reports in the literature regarding this observation. One exception is the work on astrocytes, showing that TGF-β1 induces COX-1 expression and increases PGE2 secretion (Luo et al., 1998). COX-2 is normally at very low levels or even absent from most cells; its expression can be induced by a wide variety of stimuli including inflammatory cytokines, growth factors and BK itself (Hinz and Brune, 2002; Simmons et al., 2004; Rodriguez et al., 2006). Our results demonstrate that both COX isoforms were expressed in CF, while only COX-2 was detected in CMF. This data partially agrees with Zidar et al. (2007), who found COX-2 in CMF but did not find COX-1 in CF at the infarcted myocardial zone. Thus, our results are consistent with the idea that COX-2 is expressed after cardiac damage. The role of COX isoforms in cardiovascular systems under normal and pathologic conditions is complex. In an experimental model of myocardial infarction, COX-2 inhibition showed beneficial effects on inflammation, scar formation and remodeling after myocardial infarction (LaPointe et al., 2004; Saito et al., 2004). However, other reports have suggested that COX-2 expression is induced to protect the tissue during repair (Saito et al., 2004). By bringing this evidence together, as well as the

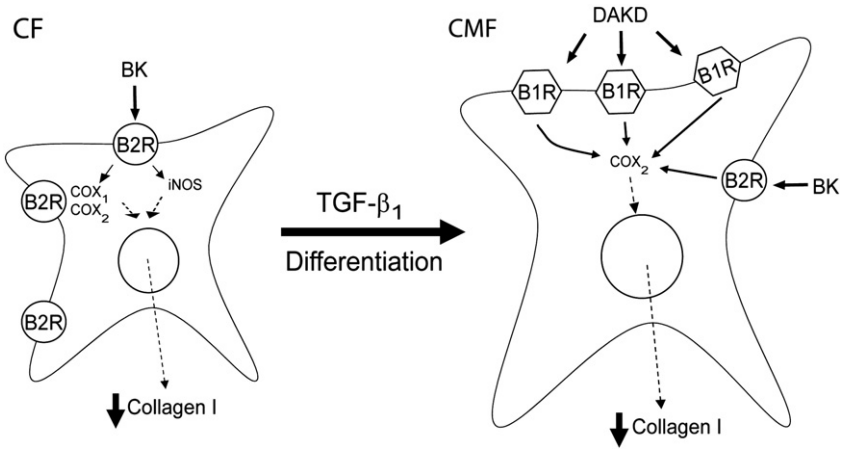

Fig. 7. Schematic representation of the effect of BK and DAKD in the present experimental systems. Left cell: cardiac fibroblasts; right cell: cardiac myofibroblasts.

differential expression observed in molecular effectors for B1R and B2R signaling pathways in CF and CMF, we suggest that B1R or B2R agonists could activate different signaling pathways involved in collagen secretion in both cell types.

We first examined the effects of BK and DAKD on collagen secretion levels. Our data showed that BK decreased collagen secretion both in CF and CMF, while DAKD only reduced collagen secretion in CMF. These results are consistent with the presence of B2R in CF and B2R and B1R in CMF. Although the mechanism by which BK decreases collagen secretion has been extensively studied in CF (Kim et al., 1999; Gallagher et al., 1998; Yu et al., 1997), no such studies have been done in CMF and the role of B1R and B2R in collagen secretion remains unexplored in CMF. Our data indicate that the decrease in collagen secretion induced by BK was blocked by the B2R antagonist HOE-140 in CF and CMF. We have also investigated the possibility that NO may mediate some or all of the responses to BK in CF and CMF. Our results indicate that L-NAME and 1400W attenuate the effect of BK on collagen secretion only in CF, suggesting the participation of NO in mediating the decrease of collagen secretion. Similar findings have been reported by Kim et al. (1999). Hence, these results are in agreement with our findings that iNOS is only present in CF. Conversely, L-NAME and 1400W had no effect on the reduction of collagen secretion induced by BK in CMF and is consistent with the absence of NOS in CMF. Consequently, these data provide evidence linking BK and NO to the reduction of collagen secretion in CF.

BK receptors are coupled to GTP-binding proteins to activate phospholipase A2 (Yu et al., 1997), resulting in an enhanced release of arachidonic acid. BK stimulates PGI2 release from rabbit CF, which significantly inhibits collagen gene expression (Gallagher et al., 1998; Yu et al., 1997). Our results indicate that indomethacin prevented the BK-induced attenuation of collagen secretion in both CF and CMF, and suggest a role of COXs in this process. This conclusion is similar to early findings described for rabbit CF (Gallagher et al., 1998). In this regard, Imai et al. (2005) found that PGE2 formation was minimally altered by BK in rabbit CF, suggesting that endoperoxides generated by BK are predominantly converted to PGI2, which acts as a negative regulator of collagen gene expression. The reduction of collagen secretion by DAKD in CMF as well as the effect of the inhibitors is in agreement with our observation that CMF differentially express COX-2, but not eNOS and iNOS, and leads us to propose that collagen secretion is mainly modulated by COX-2 in CMF.

Lastly, Fig. 7 summarizes the mechanisms by which BK, DAKD and its receptors could regulate collagen secretion in CF and CMF. Differentiation of CF to CMF induced by TGF-β1 is accompanied by parallel decreases in iNOS, COX-1 and by the induction of B1R expression. We proposed that BK decreases collagen secretion through a mechanism depending on iNOS and COXs in CF, while this takes place in a COX-2 dependent manner in CMF.

In conclusion, the present study shows that dynamic changes in B1R and B2R levels can be observed in CF and CMF. The activation of B1R and/or B2R could reduce collagen secretion and avoid excessive collagen deposition, as well as regulate cardiac remodeling after myocardial infarction. These data also support the hypothesis that the kallikrein–kinin system, acting via the B1R and/or B2R, could play a key role in protecting the heart against dysfunction and excessive remodeling.

Conflict of interest statement

None declared.

Supplementary data related to this article can be found online at doi: http://dx.doi.org/10.1016/j.taap.2012.04.013.

Acknowledgments

This work was supported by the Comisión Nacional de Ciencia y Tecnología (CONICYT), Chile [FONDECYT 1100443 to G.D.A; FONDAP 15010006 to S.L and Proyecto Anillo ACT 71, to V.V.]. M.C., I.O., R.V. and P.A., are recipients of PhD fellowships from CONICYT, and MECESUP Chile.

References

Aránguiz-Urroz, P., Soto, D., Contreras, A., Troncoso, R., Chiong, M., Montenegro, J., Venegas, D., Smolic, C., Ayala, P., Thomas, W.G., Lavandero, S., Díaz-Araya, G., 2009. Differential participation of angiotensin II type 1 and 2 receptors in the regulation of cardiac cell death triggered by angiotensin II. Am. J. Hypertens. 22, 569–576.

Balbay, Y., Tikiz, H., Baptiste, R.J., Ayaz, S., Sasmaz, H., Korkmaz, S., 2001. Circulating interleukin-1 beta, interleukin-6, tumor necrosis factor-alpha, and soluble ICAM-1 in patients with chronic stable angina and myocardial infarction. Angiology 52, 109–114.

Baum, J., Duffy, H.S., 2011. Fibroblasts and myofibroblasts: what are we talking about? J. Cardiovasc. Pharmacol. 57, 376–379.

Calixto, J.B., Medeiros, M., Fernandes, E.S., Ferreira, J., Cabrini, D.A., Campos, M.M., 2004. Kinin B1 receptors: key G-protein-coupled receptors and their role in inflammatory and painful processes. Br. J. Pharmacol. 143, 803–818.

Cheon, H., Yu, S.J., Yoo, D.H., Chae, I.J., Song, G.G., Sohn, J., 2002. Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts from rheumatoid arthritis and normal individuals. Clin. Exp. Immunol. 127, 547–552.

Diaz-Araya, G., Borg, T.K., Lavandero, S., Loftis, M.J., Carver, W., 2003. IGF-1 modulation of rat cardiac fibroblast behavior and gene expression is age-dependent. Cell Commun. Adhes. 10, 155–165.

Enquist, J., Skröde, C., Whistler, J.L., Leeb-Lundberg, L.M., 2007. Kinins promote B2 receptor endocytosis and delay constitutive B1 receptor endocytosis. Mol. Pharmacol. 71, 494–507.

Frangogiannis, N.G., 2007. Chemokines in ischemia and reperfusion. Thromb. Haemost. 97, 738–747.

Gabbiani, G., 2003. The myofibroblast in wound healing and fibrocontractive diseases. J. Pathol. 200, 500–503.
Gallagher, A.M., Yu, H., Printz, M.P., 1998. Bradykinin-induced reductions in collagen gene expression involve prostacyclin. Hypertension 32, 84–88.
Hayakawa, K., Takemura, G., Kanoh, M., Li, Y., Koda, M., Kawase, Y., Maruyama, R., Okada, H., Minatoguchi, S., Fujiwara, T., Fujiwara, H., 2003. Inhibition of granulation tissue cell apoptosis during the subacute stage of myocardial infarction improves cardiac remodeling and dysfunction at the chronic stage. Circulation 108, 104–109.
Hinz, B., Brune, K., 2002. Cyclooxygenase-2, 10 years later. J. Pharmacol. Exp. Ther. 300, 367–375.
Imai, C., Okamura, A., Peng, J.F., Kitamura, Y., Printz, M.P., 2005. Interleukin-1beta enhanced action of kinins on extracellular matrix of spontaneous hypertensive rat cardiac fibroblasts. Clin. Exp. Hypertens. 27, 59–69.
Kim, N.N., Villegas, S., Summerour, S.R., Villarreal, F.J., 1999. Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. J. Mol. Cell. Cardiol. 31, 457–466.
LaPointe, M.C., Mendez, M., Leung, A., Tao, Z., Yang, X.P., 2004. Inhibition of cyclooxygenase-2 improves cardiac function after myocardial infarction in the mouse. Am. J. Physiol. Heart Circ. Physiol. 286, H1416–H1424.
Leeb-Lundberg, L.M., Marceau, F., Muller-Esterl, W., Pettibone, D.J., Zuraw, B.L., 2005. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. Pharmacol. Rev. 57, 27–77.
Luo, J., Lang, J.A., Miller, M.W., 1998. Transforming growth factor β1 regulates the expression of cyclooxygenase in cultured cortical astrocytes and neurons. J. Neurochem. 71, 526–534.
Mathis, S.A., Criscimagna, N.L., Leeb-Lundberg, L.M., 1996. B1 and B2 kinin receptors mediate distinct patterns of intracellular Ca2+ signaling in single cultured vascular smooth muscle cells. Mol. Pharmacol. 50, 128–139.
Maurer, M., Barder, M., Bas, M., Bossi, F., Cicardi, M., Cugno, M., Howarth, P., Kaplan, A., Kojda, G., Leeb-Lundberg, F., Lötvall, J., Margerl, M., 2011. New topics in bradykinin research. Allergy 66, 1397–1406.
Moreau, M.E., Garbacki, N., Molinaro, G., Brown, N.J., Marceau, F., Adam, A., 2005. The kallikrein–kinin system: current and future pharmacological targets. J. Pharmacol. Sci. 99, 6–38.
Phagoo, S.B., Reddi, K., Anderson, K.D., Leeb-Lundberg, L.M., Warburton, D., 2001. Bradykinin B1 receptor up-regulation by interleukin-1beta and B1 agonist occurs through independent and synergistic intracellular signaling mechanisms in human lung fibroblasts. J. Pharmacol. Exp. Ther. 298, 77–85.
Pinsky, D.J., Cai, B., Yang, X., Rodriguez, C., Sciacca, R.R., Cannon, P.J., 1995. The lethal effects of cytokine-induced nitric oxide on cardiac myocytes are blocked by nitric oxide synthase antagonism or transforming growth factor beta. J. Clin. Invest. 95, 677–685.
Porter, K.E., Turner, N.A., 2009. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol. Ther. 123, 255–278.
Riad, A., Walther, T., Yang, J., Altmann, C., Escher, F., Westermann, D., Spillmann, F., Schultheiss, H.P., Tschöpe, C., 2008. The cardiovascular influence of interleukin-1 beta on the expression of bradykinin B1 and B2 receptors. Int. Immunopharmacol. 8, 222–230.
Riches, K., Hettiarachchi, N.T., Porter, K.E., Peers, C., 2010. Hypoxic remodelling of Ca2+ stores does not alter human cardiac myofibroblast invasion. Biochem. Biophys. Res. Commun. 403, 468–472.
Rodriguez, J.A., De la Cerda, P., Collyer, E., Decap, V., Vio, C.P., Velarde, V., 2006. Cyclooxygenase-2 induction by bradykinin in aortic vascular smooth muscle cells. Am. J. Physiol. Heart Circ. Physiol. 290, H30–H36.Sun, Y., Weber, K.T., 2000. Infarct scar: a dynamic tissue. Cardiovasc. Res. 46, 250–256.
Saito, T., Rodger, I.W., Hu, F., Robinson, R., Huyn, T., Giaid, A., 2004. Inhibition of COX pathway in experimental myocardial infarction. J. Mol. Cell. Cardiol. 37, 71–77.
Simmons, D.L., Botting, R.M., Hla, T., 2004. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol. Rev. 56, 387–437.
Van den Borne, S.W.M., Diez, J., Blankesteijn, W.M., Verjans, J., Hofstra, L., Narula, J., 2010. Myocardial remodeling after infarction: the role of myofibroblasts. Nat. Rev. Cardiol. 7, 30–37.
Yu, H., Gallagher, A.M., Garfin, P.M., Printz, M.P., 1997. Prostacyclin release by rat cardiac fibroblasts: inhibition of collagen expression. Hypertension 30, 1047–1053.
Zidar, N., Dolenc-Strazar, Z., Jeruc, J., Jerse, M., Balazic, J., Gartner, U., Jermol, U., Zupanc, T., Stajer, D., 2007. Expression of cyclooxygenase-1 and cyclooxygenase-2 in the normal human heart and in myocardial infarction. Cardiovasc. Pathol. 16, 300–304.