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# Expression and function of TLR4- induced B1R bradykinin receptor on cardiac fibroblasts

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ABSTRACT

Cardiac fibroblasts (CF) are key cells for maintaining extracellular matrix (ECM) protein homeostasis in the heart, and for cardiac repair through CF-to-cardiac myofibroblast (CMF) differentiation. Additionally, CF play an important role in the inflammatory process after cardiac injury, and they express Toll like receptor 4 (TLR4), B1 and B2 bradykinin receptors (B1R and B2R) which are important in the inflammatory response. B1R and B2R are induced by proinflammatory cytokines and their activation by bradykinin (BK: B2R agonist) or des-arg-kallidin (DAKD: B1R agonist), induces NO and PGI2 production which is key for reducing collagen I levels. However, whether TLR4 activation regulates bradykinin receptor expression remains unknown. CF were isolated from human, neonatal rat and adult mouse heart. B1R mRNA expression was evaluated by qRT-PCR, whereas B1R, collagen, COX-2 and iNOS protein levels were evaluated by Western Blot. NO and PGI2 were evaluated by commercial kits. We report here that in CF, TLR4 activation increased B1R mRNA and protein levels, as well as COX-2 and iNOS levels. B1R mRNA levels were also induced by interleukin-1α via its cognate receptor IL-1R1. In LPS-pretreated CF the DAKD treatment induced higher responses with respect to those observed in non LPS-pretreated CF, increasing PGI2 secretion and NO production; and reducing collagen I protein levels in CF. In conclusion, no significant response to DAKD was observed (due to very low expression of B1R in CF) – but pre-activation of TLR4 in CF, conditions that significantly enhanced B1R expression, led to an additional response of DAKD.

## 1. Introduction

Cardiac fibroblasts (CF) are a key cell for maintaining extracellular matrix (ECM) protein homeostasis in heart tissue, and also for cardiac repair through CF-to-cardiac myofibroblast (CMF) differentiation (Porter and Turner, 2009). Moreover, CF play a sentinel role responding to mechanical and chemical stimuli by releasing cytokines and chemokines which impact directly on resident cardiac cells and also on infiltrating immune cells (Pinto et al., 2016; Díaz Araya et al., 2015).

Toll-like receptors (TLR) recognize and react to highly conserved motifs known as pathogen-associated microbial patterns (PAMPs, like LPS), or to damage-associated molecular patterns (DAMPs or "alarmins") (Chen and Frangogiannis, 2013; Frantz et al., 1999). TLR4 is one of the most studied isoforms of this family of receptors and its activation is a key element in the initiation and resolution of inflammatory responses by many PAMPs and DAMPs (Sabroe et al., 2008). These stimuli promote a strong proinflammatory response characterized by the release of cytokines, chemokines and expression of cellular adhesion molecules (ICAM and VCAM) (Humeres et al., 2016; Boza et al., 2016). CF are able to respond to DAMPs in the damaged heart (Turner, 2016). In addition, CF appear to be the main source of the proinflammatory cytokine interleukin-1 (IL-1), which has two distinct gene products (IL-1α and IL-1β) with indistinguishable biological activities that mediate their effects through IL-1 receptor 1 (IL1R1)

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activation (Maqbool et al., 2013; Turner, 2014). IL-1α is only released from damaged or necrotic cells; however, it has been widely recognized and described as a DAMP that triggers the innate immune response (Turner, 2016). IL-1α is expressed by cardiac myocytes and CF with increased levels in infarcted myocardium (Turner et al., 2007). This cytokine has been shown to induce distinct patterns of ECM proteins and/or protease expression in the heart, contributing to adverse remodeling in heart failure (Maqbool et al., 2013). IL1R1 signaling pathways have significant similarity with TLR signaling (Frangogiannis, 2008). Once activated, both TLR and IL1R1 receptors result in stimulation of many signaling pathways including NFκB, JNK, p38 and ERK, which lead to transcription of proinflammatory cytokines such as IL-1β, IL-6 and monocyte chemoattractant protein 1 (MCP-1) (Turner et al., 2009; Boza et al., 2016; Humeres et al., 2016); however, whether TLR4 or IL1R1 activation increases kinin receptor expression in CF is unknown.

Kinins are described as controllers of many cardiovascular effects and between them as antagonists of the renin-angiotensin-aldosterone system (RAAS), leading to vascular dilation and increased vascular permeability. In addition, kinins decrease ECM protein production leading to reduced adverse myocardial remodeling (Leeb-Lundberg et al., 2005). Furthermore, kinins play an important role in processes that accompany inflammation, as well as tissue damage and repair (McLean et al., 2000). These effects are mediated by the activation of B1 and B2 kinin receptors (B1R and B2R). Under physiological conditions, B2R (which is expressed in a constitutive manner) is the main receptor responsible for the action of kinins (Marceau et al., 1997). On the other hand, only a few cell types express B1R (which is expressed in an inducible manner), and this receptor is increased in pathological conditions that occur with inflammation such as ischemia, atheromatous disease or exposure to inflammatory cytokines (Leeb-Lundberg et al., 2005; Regoli and Barabé, 1980; Marceau et al., 1998). The presence of B2R has been described in rat and human CF (Villarreal et al., 1998), and B1R expression was described in rat CF and CMF; however, B1R levels were higher in CMF compared to CF, while B2R expression did not change between both cells (Catalán et al., 2012). Bradykinin (BK) is a B2R agonist, while des-Arg-KD (DAKD) is a B1R agonist (Ju et al., 2000). B1R and B2R are coupled to G proteins (Gαi/Gαq), activating different signaling pathways leading to production of NO and prostaglandins (PGs) E2 and I2, respectively.

Currently, there is no direct evidence available relating TLR4 activation with B1R expression in CF and CMF. Our previous findings demonstrated the presence of B1R in CF and CMF, being much higher in CMF; and also that iNOS is present in CF but not in CMF; however, COX-2 is present only in CMF (Catalán et al., 2012). Also, we determined that PGI2 and NO are released by CF and CMF, which had shown an antifibrotic effect due to their capacity to reduce collagen secretion (Catalán et al., 2012). However, a relationship between TLR4 or IL1R1 activation and B1R expression in CF and CMF has not yet been established. Thus to focus on TLR4 and B1R activation could be relevant in cardiac damage where acute cardiac inflammation is necessary to initiate wound healing. With all these antecedents, we propose that TLR4 activation induces B1R expression in CF, and therefore enhances kinin effects on the reduction of collagen I synthesis.

## 2. Materials and methods

### 2.1. Materials

B1R and GAPDH human and mouse primers were obtained from Applied Biosystems. B1R and B2R primary antibodies were purchased from Enzo Life Science. TGF-β and TNF-α were obtained from Millipore. Trypan blue, Bradford solution, primary antibodies (α-smooth muscle actin [α-SMA], β-tubulin and GAPDH), kinin agonists BK and DAKD, inhibitors for ERK pathway (PD98059), JNK (SP600125), p38 (SB202190), PI3K (LY294002), NF-κB pathway (IMD-0354), TGF-R (SB431542) and TNFα-R (SPD304) were acquired from Sigma Aldrich. 488 Alexa Fluor®-conjugated secondary antibody was obtained from Life Technologies. Trypsin/EDTA, prestained molecular weight standard, fetal bovine serum (FBS) and fetal calf serum (FCS) were from Gibco BRL. All organic and inorganic compounds were from Merck and Winkler. The enhanced chemiluminescence (ECL) reagent was from Perkin Elmer Life Sciences. Sterile plastic cell culture materials were from Costar® and Corning® (Merck). Primary antibodies (TLR4, COX-1, COX-2, iNOS) were purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated with horseradish peroxidase were from Calbiochem. Pro-collagen I antibody was purchased from Abcam. TAK-242 was from Invivogen. LPS ultrapure and IL-1α (human recombinant) were from Invitrogen. The ELISA kit for 6-keto prostaglandin $F_{1\alpha}$ and fluorescence kit for NO were acquired from Cayman Chemical Company.

### 2.2. Cardiac fibroblast isolation, culture and treatments

Cardiac fibroblasts from multiple species were studied to identify responses and mechanisms that were common across species and hence of general biological importance.

#### 2.2.1. Murine CF

IL1R1 knockout (KO) mice were established by crossing female PGK-Cre global deleter mice (Jackson Labs) (Lallemand et al., 1998), with male mice expressing a modified IL1R1 gene flanked by loxP sites (E. Pinteaux, University of Manchester) (Abdulaal et al., 2016), on a C57BL/6 background. All animal procedures were carried out in accordance with the Animal Scientific Procedures Act (UK) 1986 and the University of Leeds Animal Welfare and Ethical Review Committee. CF were isolated from wild-type (WT) and IL1R1 KO mice and cultured as described previously (Mylonas et al., 2017). In brief, mice were terminally anesthetized with saline containing ketamine at 50 mg/kg and medetomidine at 0.5 mg/kg by i.p. injection. Heart tissue was digested before fibroblast isolation. Briefly, infarct and surrounding border heart tissues were chopped into small pieces and digested in collagenase D and DNase 1 (2.5 mg/mL collagenase D; 60 U/mL DNAse 1; Ambion) in HBSS (GIBCO; Thermo Fisher) at 37 °C for 30 min following dissociation by gentleMacs Dissociator (according to manufacturer's instructions; Miltenyi; Surrey, UK). The digested tissue was gently disaggregated and filtered through a 30 μm cell strainer to remove larger cells (including cardiomyocytes). Cells were then centrifuged at 300*g* for 5 min and washed in PBS. Cardiac fibroblasts were isolated magnetically using a Miltenyi neonatal cardiac fibroblast isolation kit (MACS), according to manufacturer's instructions. Experiments were performed on cells at passage number 2. Cells were cultured in 6-well plates and serum starved for 24 h prior to stimulation.

#### 2.2.2. Human CF

Human CF were isolated from biopsies of right atrial appendage from patients without left ventricular dysfunction undergoing coronary artery bypass surgery at the Leeds General Infirmary. Local ethical committee approval (reference number: 01/040) and informed patient consent were obtained. Cell were isolated according to Turner et al., 2003. Heart tissue was minced and digested by incubation with 800 U/mL collagenase type II solution (Worthington Biochemical Corporation, Lakewood, NJ, USA) in Dulbecco's modified Eagle medium (DMEM) containing 0.05% bovine serum albumin (BSA) for 4 h at 37 °C. Cells were pelleted by centrifugation, washed with DMEM/BSA, and resuspended in full growth medium (DMEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 μg/mL penicillin G, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin). Cells were plated into cell culture flasks for 30 min to allow fibroblasts to adhere. Following removal of non-adherent cells, fibroblasts were cultured to confluence in fresh full growth medium in a humidified atmosphere of 5% CO2 in air at 37 °C, and subsequently passaged by trypsinization. Experiments were

performed on early passage cells (2–5) from up to 15 different patients. Cells were cultured in 6-well plates and serum starved for 24 h prior to stimulation.

#### 2.2.3. Rat CF

Sprague Dawley rats were obtained from the Animal Breeding Facility of the School of Chemical and Pharmaceutical Sciences at the University of 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 the University of Chile Institutional Ethics Review Committee. CF were isolated from 2 or 3-day-old Sprague-Dawley rats and cultured as described previously (Boza et al., 2016). Briefly, the neonatal rats were decapitated and their hearts were extracted under aseptic environment. Atria were removed and ventricles were cut into small pieces (~1–2 mm) for posterior collagenase II digestion. The digestion yield was separated by 10 min centrifugation at 1000 rpm. The pellet was resuspended in 10 mL of DMEM-F12 supplemented with 10% FBS and antibiotics (100 μg/mL streptomycin and 100 units/mL penicillin) and cultured in a humidified atmosphere of 5% CO2 and 95% O2 at 37 °C until confluence (5 days). The purity of the CF population was assessed through the expression of several markers. Cardiac fibroblasts had positive staining for vimentin (Santa Cruz Biotech, CA), while being negative for sarcomeric actin and desmin (Sigma Chemical Co, St Louis, Mo). Experiments were performed on cells at passage 2. Cells were cultured in 35 mm well plates and serum starved for 24 h prior to stimulation.

Rat CF were stimulated with LPS (1 μg/mL) or TNF-α for different experimental times (0–72 h). For the inhibition experiments, cells were pre-incubated with TAK-242 (TLR4 inhibitor, 4 μM), SPD304 (TNFα-R inhibitor, 5 μM), and SB431542 (TGF-R inhibitor, 10 nM) for 1 h before LPS/TNF-α incubation. To determine collagen I protein levels, $PGI_2$ secretion and NO production, CF were treated with LPS for 48 h to induce an increase in B1R levels. After that, the medium was removed, cells washed 3 times with 2 mL PBS and then stimulated with 100 nM DAKD or 100 nM BK.

### 2.3. Differentiation of cardiac fibroblasts into cardiac myofibroblasts

Primary rat CF cultures were stimulated with TGF-β1 (5 ng/mL) for 96 h and characterized as CMF (α-SMA-positive). Then culture medium was changed without TGF-β1 and cells were stimulated with respective agonist or inhibitors.

### 2.4. Determination of B1R mRNA levels by qRT-PCR

RNA was extracted from CF (Aurum Total RNA kit) following appropriate treatments. Real time RT-PCR was performed using human (Hs00664201_s1) or mouse (Mm04207315_s1) B1R primers and Taqman probes (Applied Biosystems), as described previously (Turner et al., 2009). Data are normalized to expression of human (Hs99999905_m1) or mouse (Mm99999915_g1) GAPDH endogenous control mRNA expression and expressed relative to control using the formula $2^{-\Delta\Delta CT}$.

### 2.5. Western blot

After incubation, cells were extracted with a protease inhibitor cocktail-containing lysis buffer. Lysates were vigorously vortexed for 10 s and centrifuged at 15000 rpm for 10 min, and total protein content was determined using Bradford assay. Equivalent amounts of protein were subjected to SDS-PAGE. Western blotting was performed by transferring proteins to nitrocellulose membranes and blocking with 10% fat-free milk (*w/v*) in TBS-Tween for 1 h at room temperature. Membranes were probed with the appropriate primary antibody: α-SMA (1:5000), B1R (1:1000), B2R (1:1000), iNOS (1:1000), collagen I (1:1000), COX-1 (1:1000), COX-2 (1:1000) and β-tubulin (1:1000) overnight at 4 °C and then with peroxidase-conjugated secondary antibody for 2 h at room temperature. Finally, the ECL Advance Western Blotting Detection Kit was used for immunodetection. Protein levels were determined by densitometric analysis using Image J (NIH, Bethesda, MD, USA) and normalized to the corresponding GAPDH or β-tubulin levels.

### 2.6. Immunofluorescence assay

CF were fixed in 4% paraformaldehyde solution for 20 min at room temperature and permeabilized in 0.1% Triton × 100 for 10 min at room temperature. Non-specific proteins were blocked with 3% bovine serum albumin solution for 30 min at room temperature. Cells on coverslips were incubated with B1R and α-SMA antibodies overnight at 4 °C and an appropriate fluorophore-conjugated secondary antibody for 2 h at room temperature. Images were obtained using a confocal microscope and processed with Image J/Fiji software.

### 2.7. Determination of $PGI_2$ secretion by ELISA assay

$PGI_2$ levels were estimated by measuring its metabolite, $PGF_{1\alpha}$. Cell culture medium was collected and analyzed using the $PGF_{1\alpha}$ ELISA kit according to the manufacturer's protocol.

### 2.8. Determination of NO levels by fluorescence assay

Nitric oxide (NO) levels were measured in cell culture ($10^4$ cells/well) on a 96 well plate, by detection of fluorescein at excitation and emission wavelengths of 485 and 535 nm respectively, according to the kit manufacturer's instructions.

### 2.9. Statistical analysis

Data are presented as mean ± SEM from at least four independent experiments. Statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons, and two-way ANOVA with Bonferroni post hoc test with GraphPad Prism 5.0 software. $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. IL1R1 activation through PI3K/Akt increases B1R mRNA levels in cardiac fibroblasts

CF from wild-type (WT) and IL1R1 knockout (KO) mice were treated with IL-1α and B1R mRNA levels measured. The results in Fig. 1A show that treatment with IL-1α increased B1R mRNA levels in a statistically significant manner only in the WT cardiac fibroblasts at both treatment times (2 and 6 h), and no effects were observed in ILR1KO cardiac fibroblasts, as expected. Furthermore, in human CF (Fig. 1B), IL-1α strongly induced B1R mRNA, reaching an increase of 20-fold at 2 and 6 h compared to control CF. To determine which signaling pathways were involved in this increase, we incubated human CF with p38-MAPK, PI3K/Akt and NFκB pathway inhibitors 30 min prior to adding IL-1α. Results showed that the PI3K inhibitor LY294002 and the p38-MAPK inhibitor SB203580 reduced the increase in B1R mRNA levels induced by IL-1α (Fig. 1C).

### 3.2. TLR4 activation through PI3K/Akt increases B1R mRNA levels in cardiac fibroblasts

CF from WT and IL1R1 knockout (KO) mice were treated with LPS (TLR4 agonist) and B1R mRNA levels were measured. In Fig. 2A the results show that LPS increased B1R mRNA levels in both sets of cardiac fibroblast (WT and IL1RKO), with a trend towards higher expression at

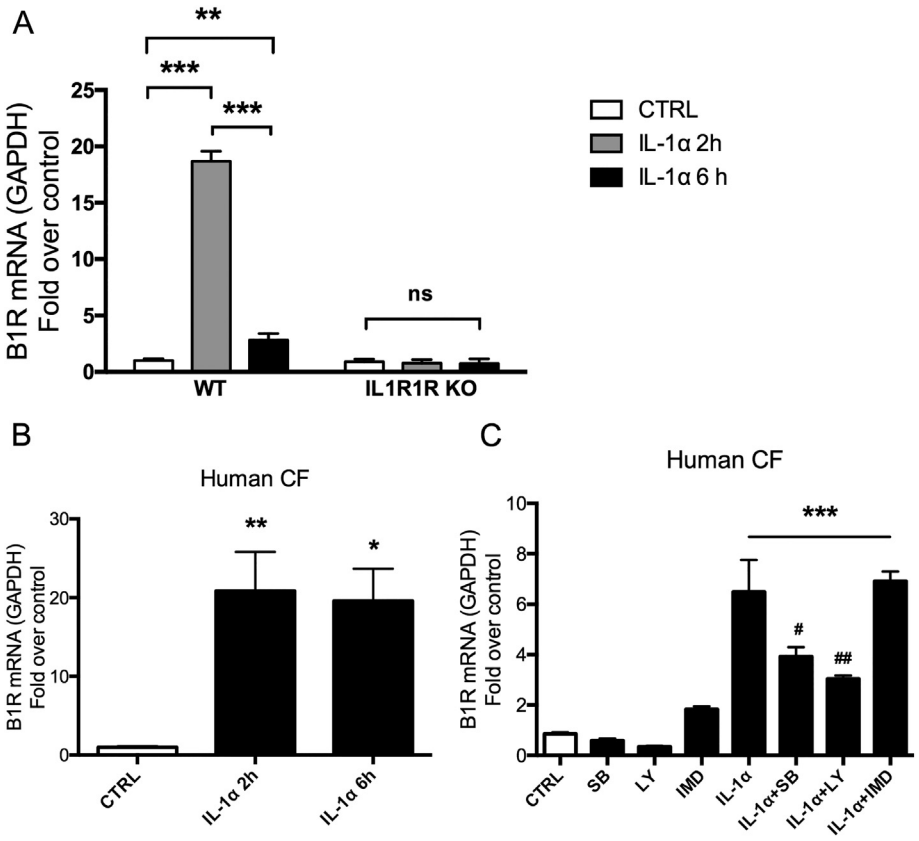


**Fig. 1.** IL-1α increases B1R mRNA expression. (A-B) WT mouse CF (A), IL1R1 KO mouse CF (A) or human CF (B) were incubated for 2 and 6 h with 0.1 μg/mL IL-1α before measuring B1R mRNA levels by RT-PCR. (A)**p < 0.01, ***p < 0.001. (B) *p < 0.05, **p < 0.01 vs CTRL. (C) Human CF were incubated for 1 h with inhibitors of p38 MAPK (10 μM SB203580; SB), PI3K (10 μM LY294002; LY) and IKK2 (10 μM IMD-0354; IMD) before incubation for 2 h without or with 0.1 μg/mL IL-1α. ***p < 0.001 vs CTRL, #p < 0.05 ##p < 0.01 vs IL-1α. Data are mean ± SEM of 4 independent experiments.

early times for the WT cardiac fibroblasts and at later times for the ILR1RKO cardiac fibroblasts. A two-way ANOVA indicated that the results are time-dependent, but not cell type-dependent i.e. there is no significant difference in time courses between the two sets of cells. In human CF, LPS increased B1R mRNA levels significantly compared to control (Fig. 2B). To determine which signaling pathways were

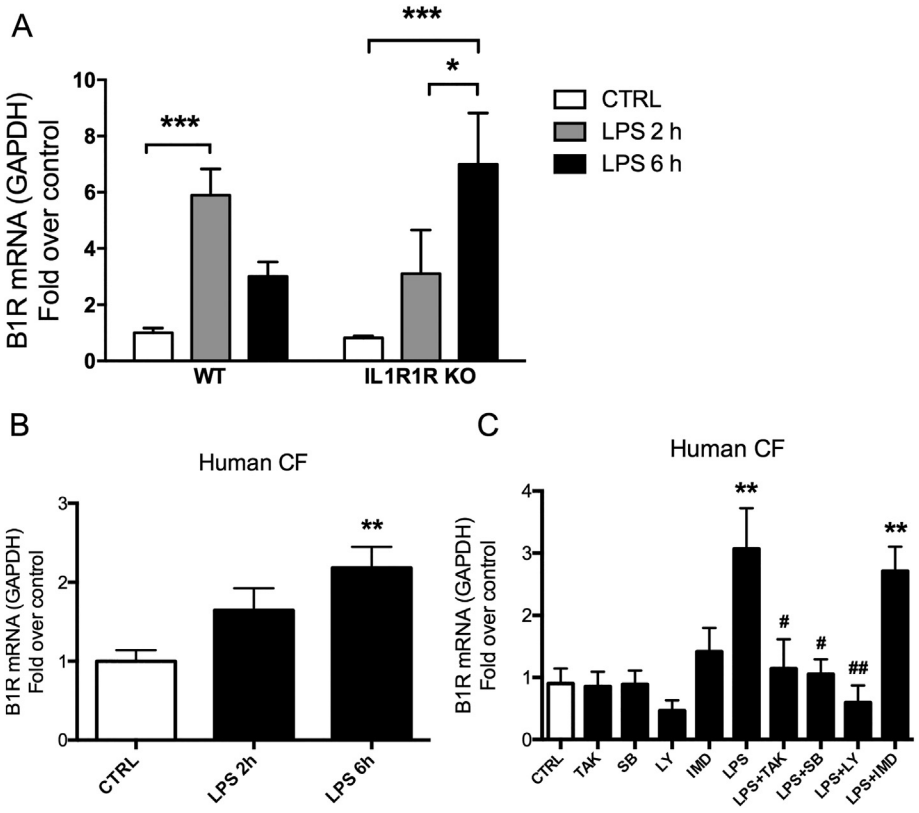


**Fig. 2.** TLR4 activation increases B1R mRNA expression. (A-B) WT mouse CF (A), IL1R1 KO mouse CF (A) or human CF (B) were incubated for 2 and 6 h with 1 μg/mL LPS before measuring B1R mRNA levels by RT-PCR. (A) *p < 0.05, ***p < 0.001. (C) Human CF were incubated for 1 h with inhibitors of TLR4 (4 μM TAK-242; TAK), p38 MAPK (10 μM SB203580; SB), PI3K (10 μM LY294002; LY) and IKK2 (10 μM IMD-0354; IMD) before incubation for 2 h without or with 1 μg/mL LPS. **p < 0.01 vs CTRL. #p < 0.05, ##p < 0.01 vs LPS. Data are mean ± SEM of 4 independent experiments.

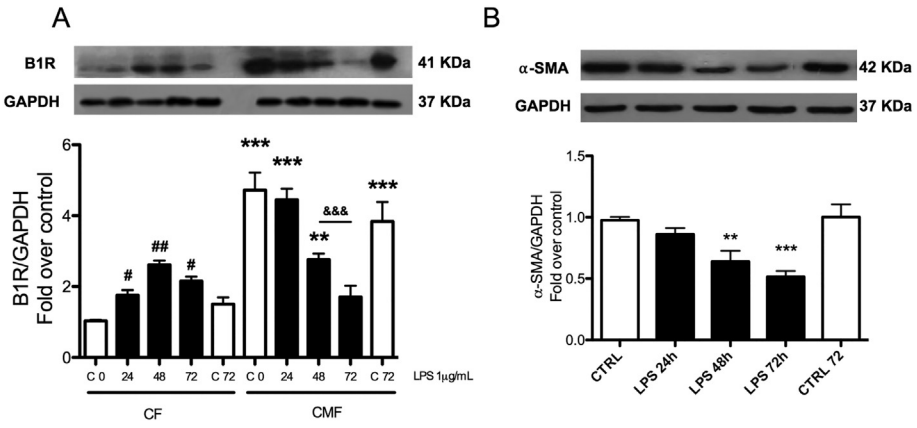


**Fig. 3.** TLR4 activation increases B1R protein levels in CF and reduces B1R protein levels in CMF. (A) Rat CF or CMF (CF treated with TGF-β for 96 h) were incubated with 1 μg/mL LPS for 24, 48 and 72 h. B1R protein levels were measured by WB, using GAPDH as a loading control. **p < 0.01; ***p < 0.001 vs C0 (CF); #p < 0.05; ##p < 0.01 vs C0 (CF); &&& p < 0.001. Data are mean ± SEM of 5 independent experiments. (B) Rat CMF were incubated with 1 μg/mL LPS for 24, 48 and 72 h. α-SMA protein levels were measured by WB, using GAPDH as a loading control. **p < 0.01; ***p < 0.001 vs CTRL. Data are mean ± SEM of 4 independent experiments.

important, and whether TLR4 activation was responsible for this increase, we incubated human CF with p38-MAPK, PI3K/Akt, NFκB and TLR4 inhibitors (Fig. 2C). Results showed that both LY294002 (PI3K inhibitor) and SB203580 (p38-MAPK inhibitor), as well as TAK-242 (TLR4 inhibitor), prevented the increase induced by LPS.

### 3.3. TLR4 activation increases B1R protein levels in cardiac fibroblasts

To establish if the increase in B1R mRNA levels led to an increase in protein levels, rat CF were stimulated for 24, 48 and 72 h with LPS. Western blotting indicated that LPS was capable of inducing an increase in B1R protein levels (Fig. 3A). The maximum increase in B1R protein levels of 3-fold compared to non-stimulated fibroblasts was reached after 48 h LPS stimulation, and at 72 h the increase remained significant. In contrast, in myofibroblasts, the control condition had the highest B1R protein levels (in accord with other reports, as B1R is highly induced by inflammatory/profibrotic events). Surprisingly LPS decreased B1R protein levels significantly compared to CMF control, suggesting an anti-fibrotic role of TLR4 activation. Moreover, LPS also decreased α-SMA protein levels (Fig. 3B), suggesting TLR4 activation was opposing myofibroblast differentiation.

To evaluate the role of TLR4 activation in LPS-induced B1R protein levels, rat CF were incubated with TAK-242 (TLR4 inhibitor) and LPS (Fig. 4A). TAK-242 prevented the increase of B1R protein levels induced by LPS at all times, confirming that TLR4 activation is essential for LPS-induced B1R expression (Fig. 4A). To discard any effect of autocrine secretion of TGF-β in culture on B1R expression levels, SB431542 (TGF-R inhibitor) was added 1 h prior to treatment with LPS, and we performed immunocytochemistry to evaluate B1R localization and α-SMA levels and assembly (Fig. 4B). As expected, LPS increased the amount of B1R protein (green staining) and decreased α-SMA fibers (red staining); in addition, CF were larger in the 48 h control compared to the 0 h control demonstrating an autocrine effect of TGF-β secretion into the culture media, leading to CF differentiation to CMF (Fig. 4B). A 3D image reconstruction of 9 slices was performed on the LPS + SB431542 (48 h) sample to establish the localization of B1R. It was located mainly in the nucleus and cytosol, but not the cell membrane (Fig. 4C).

### 3.4. TLR4 activation increases COX-2 protein levels in CF and CMF

COX-2 is an inducible enzyme responsible for the conversion of arachidonic acid into prostaglandins like $PGI_2$, and is a potent vasodilator. Furthermore, COX-2 is downstream in the kinin signaling pathway. This led us to hypothesize that there was a relationship between TLR4 and COX2. As in previous experiments, CF and CMF were incubated with LPS and TAK-242 to determine COX-2 protein levels. We corroborated that COX-2 was poorly expressed in control fibroblasts (Fig. 5A) compared to control myofibroblasts (Fig. 5B) which had higher protein levels. Furthermore, LPS induced an increase in COX-2 protein levels in CF and CMF in a time-dependent manner, following a similar profile (Fig. 5A, B). This was prevented by the TLR4 inhibitor TAK-242 in both CF and CMF (Fig. 5A, B).

### 3.5. TLR4 activation increases $PGI_2$ secretion

COX enzymes convert arachidonic acid into thromboxane A2 and prostaglandins. $PGI_2$ (prostacyclin) is produced by COX-2 and is highly induced by LPS and kinins. To determine if the increase in COX-2 levels due to TLR4 activation (Fig. 5A, B) had an effect on $PGI_2$ secretion, we stimulated CF with LPS in a time-dependent manner. LPS induced a statistically significant increase of 6-keto-$PGF_{1\alpha}$ (stable hydrolyzed product of $PGI_2$) at all times, reaching the highest level of secretion (2-fold) at 48 h post-stimulation (Fig. 5C). To corroborate if the increase in $PGI_2$ secretion was dependent on TLR4 activation, we incubated CF with TAK-242, and showed that TAK-242 prevented the increase of $PGI_2$ secretion induced by LPS (Fig. 5D).

### 3.6. TLR4 activation increases iNOS protein levels and NO secretion in CF and CMF

To evaluate the influence of TLR4 activation on iNOS expression, CF were incubated with LPS and/or TAK-242 for 24–72 h. LPS treatment increased iNOS protein levels at all times of treatment, reaching its highest levels at 48 h, and this effect was completely prevented by TAK-242 (Fig. 6A). Previously, we demonstrated that CMF did not express iNOS under basal conditions (Catalán et al., 2012). We confirmed this (Fig. 6B), but also observed that LPS acted as a high inducer of iNOS protein levels in CMF in a time-dependent manner, reaching a maximum increase of 15-fold at 72 h, and this effect was also completely prevented by TAK-242 (Fig. 6B).

As a consequence of the increase in iNOS protein levels, we proceeded to verify that this enzyme was capable of producing increased levels of NO. We incubated CF with LPS, TAK-242 and L-NAME (inhibitor of NOS) to measure NO production by a fluorometric assay. As expected, LPS increased NO secretion in CF and this effect was prevented by TAK-242 and L-NAME (Fig. 6C).

### 3.7. In cardiac fibroblasts the pre-activation of TLR4 enhances kinin effects on prostacyclin and NO secretion

To evaluate if there was any synergy between TLR4 and B1R activation in regulating $PGI_2$ and NO secretion, CF pretreated with LPS for 48 h (to induce B1R) were stimulated with BK or DAKD for a further 24 h. BK activates B2R and by itself was capable of increasing $PGI_2$ secretion into the culture media to the same magnitude as LPS (Fig. 7A). Co-incubation of LPS and BK did not induce a further increase in $PGI_2$ secretion (Fig. 7A). With DAKD, $PGI_2$ secretion was also increased;

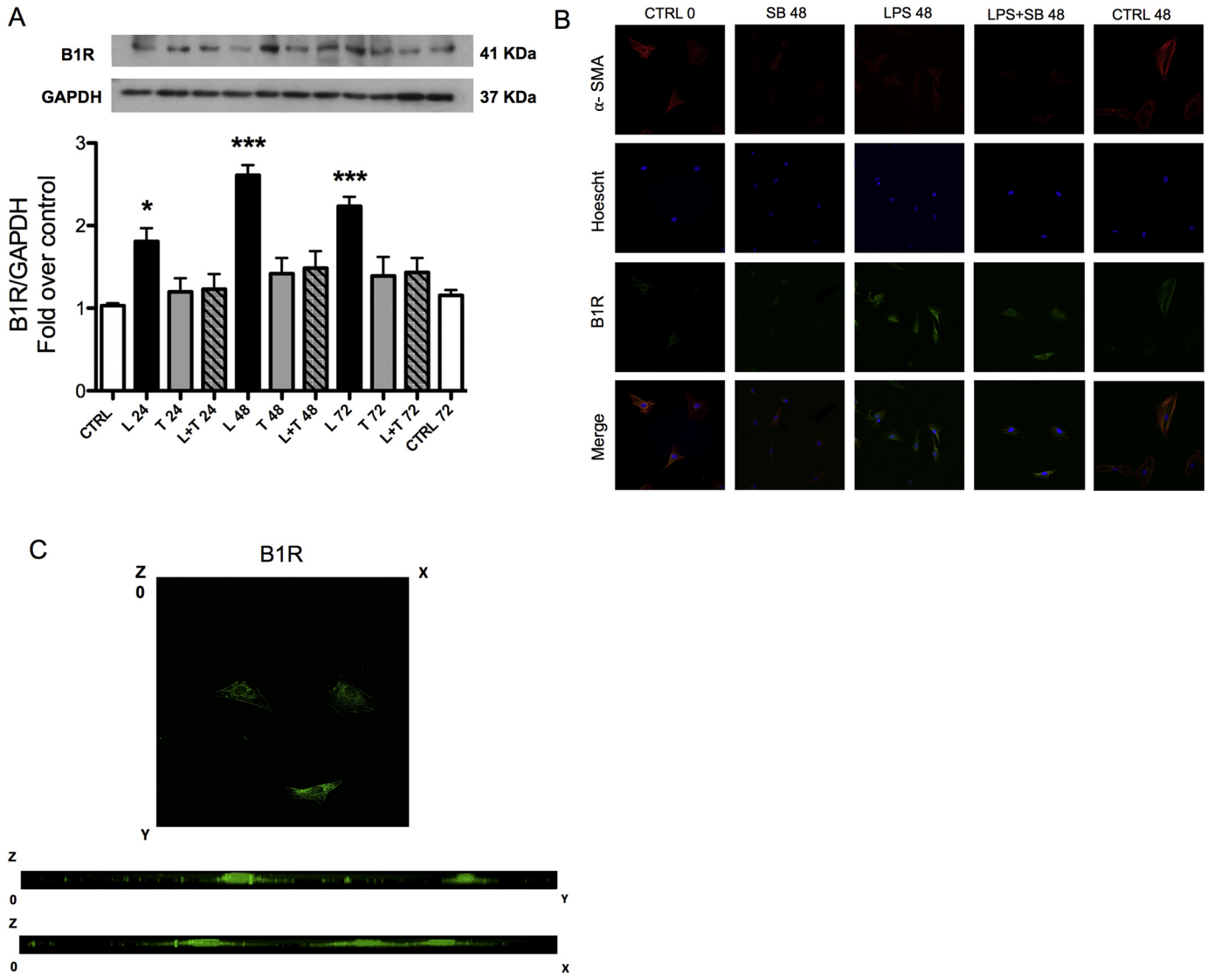


**Fig. 4.** TLR4 activation increases B1R protein levels in CF. (A) Rat CF were pretreated for 30 min with 10 nM SB431542 (TGF-R inhibitor), followed by incubation with 1 μg/mL LPS (L), 2 μM TAK-242 (T) or both together for 24, 48 and 72 h. B1R protein levels were measured by WB. *p < 0.05; **p < 0.01; ***p < 0.001 vs CTRL. Data are mean ± SEM of 6 independent experiments. (B) ICC of CF incubated with 1 μg/mL LPS without or with 10 nM SB431542 (SB) for 48 h. B1R was detected by immunofluorescence using anti-B1R antibody and Alexa Fluor® 488-conjugated secondary antibody (green staining). α-SMA was detected using anti-α-SMA antibody and Alexa Fluor® 566-conjugated secondary antibody (red staining). (C) 3D *Z*-stack reconstruction of 9 slices of LPS + SB 48 sample. X, Y and Z represents the axis in Cartesian coordinate system. 0 is the origin point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

however, the effect of LPS together with DAKD was larger than with DAKD or LPS alone (Fig. 7B).

B2R activation with BK induced a significant increase of NO (approximately 1.5-fold over control) (Fig. 7C) whereas the apparent LPS-induced increase was not statistically significant. The effect of LPS and BK together was higher in magnitude than LPS or BK alone (> 2-fold over control) (Fig. 7C). Analysis of these results by two-way ANOVA showed that there was no interaction between LPS and BK. Contrary to BK, DAKD was unable to induce an increase of NO production, but LPS and DAKD together stimulated NO production to a higher level than LPS or DAKD alone (Fig. 7D).

### *3.8. In cardiac fibroblasts the pre-activation of TLR4 enhances collagen I reduction induced by B1R activation*

To evaluate B1R capacity as an anti-fibrotic receptor, we measured pro-collagen I protein levels (Fig. 8). DAKD reduced pro-collagen I protein levels marginally by itself. LPS pretreatment reduced pro-collagen I protein levels by almost 75% compared to control. In LPS-pretreated CF, DAKD also induced a further reduction in pro-collagen-I levels, but this effect was largely masked by the effect of LPS (Fig. 8). Taken together, these results suggest an anti-fibrotic effect of B1R activation that is enhanced by TLR4 activation.

## 4. Discussion

### *4.1. TLR4 and IL1R1 activation increase B1R mRNA expression in cardiac fibroblasts*

Our results showed that TLR4 and IL1R1 activation play a key role in B1R mRNA expression. We stimulated CF with IL-1α which is a known DAMP and also with LPS which is a classical PAMP, and both are key mediators of sterile and non-sterile inflammation. Previous data from our laboratory had shown that CF can respond in an efficient

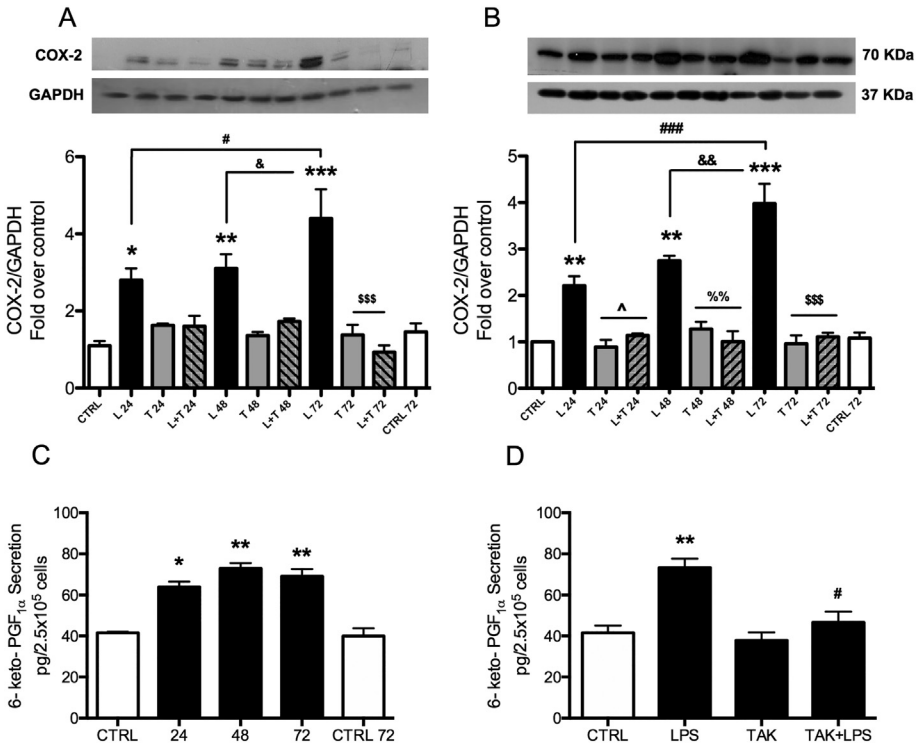


**Fig. 5.** TLR4 activation increases COX2 protein levels in CF and CMF, increasing $PGI_2$ secretion. (A, B) Rat CF (A) or CMF (B) were incubated with 1 μg/mL LPS (L), 2 μM TAK-242 (T) or both together for 24, 48 and 72 h before measuring COX-2 protein levels by WB with GAPDH as loading control. *p < 0.05; **p < 0.01; ***p < 0.001 vs CTRL. #p < 0.05; ###p < 0.001 vs L 24. &p < 0.05; &&p < 0.01 vs L 48. %%p < 0.01 vs L 48. $$$p < 0.001 vs L 72. ^p < 0.05 vs L 24. (C) Rat CF were stimulated with 1 μg/mL LPS for 24, 48 and 72 h before measuring $PGI_2$ secretion by EIA kit assay. *p < 0.05; **p < 0.01 vs 0 h. (D) Rat CF were incubated with 1 μg/mL LPS, 2 μM TAK-242 or both together for 48 h before measuring $PGI_2$ secretion by EIA kit assay. *p < 0.05 vs C; #p < 0.05 vs LPS. Data are mean ± SEM of 5 independent experiments.

manner to LPS through TLR4 increasing cytokine and chemokine expression (Humeres et al., 2016); and also, previous findings had shown that IL-1α is expressed by cardiac myocytes and fibroblasts (Maqbool et al., 2013). Here our results demonstrated that IL-1α is a potent inducer of B1R expression in human, mouse and rat CF and that IL1R1 was essential for that increase as it was absent in CF from IL1R1 KO mice. Both TLR4 and IL1R1 receptors are associated with many adaptor proteins, including MyD88 (Lu et al., 2008), and our results may therefore suggest an important role for MyD88 in coupling inflammatory signals to B1R expression.

Furthermore, TLR4 and IL1R1 activation involves NFκB, PI3K/AKT, p38 MAPK and ERK1/2 signaling pathways. In this respect, our findings suggest that the increase in B1R mRNA is regulated by PI3K/Akt and p38 MAPK activation in response to either IL-1α or LPS. Similar to our findings, in rabbit aortic smooth muscle cells, Larrivé et al. suggested that B1R expression was regulated by p38 MAPK (Larrivé et al., 1998). Other studies have shown that B1R expression can be regulated by TNF-α and IL-1β (Haddad et al., 2000; Moreau et al., 2007). With respect to

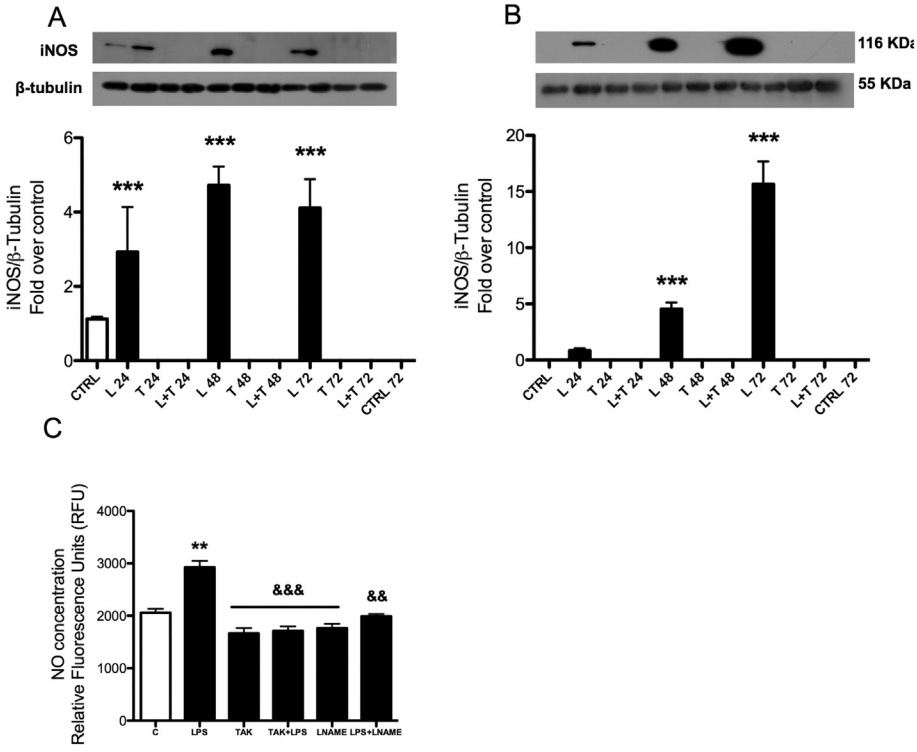


**Fig. 6.** TLR4 activation increases iNOS protein levels in CF and CMF, increasing NO production. (A, B) Rat CF (A) or CMF (B) were incubated with 1 μg/mL LPS (L), 2 μM TAK-242 (T) or both together for 24, 48 and 72 h before measuring iNOS protein levels by WB with β-tubulin as loading control. ***p < 0.001 vs CTRL. (C) Rat CF were stimulated with 1 μg/mL LPS, 4 μM TAK-242 or 10 μM L-NAME for 24 h before measuring NO production by immunofluorescence assay. **p < 0.01 vs C. &&&p < 0.001 vs LPS. Data are mean ± SEM of 5 independent experiments.

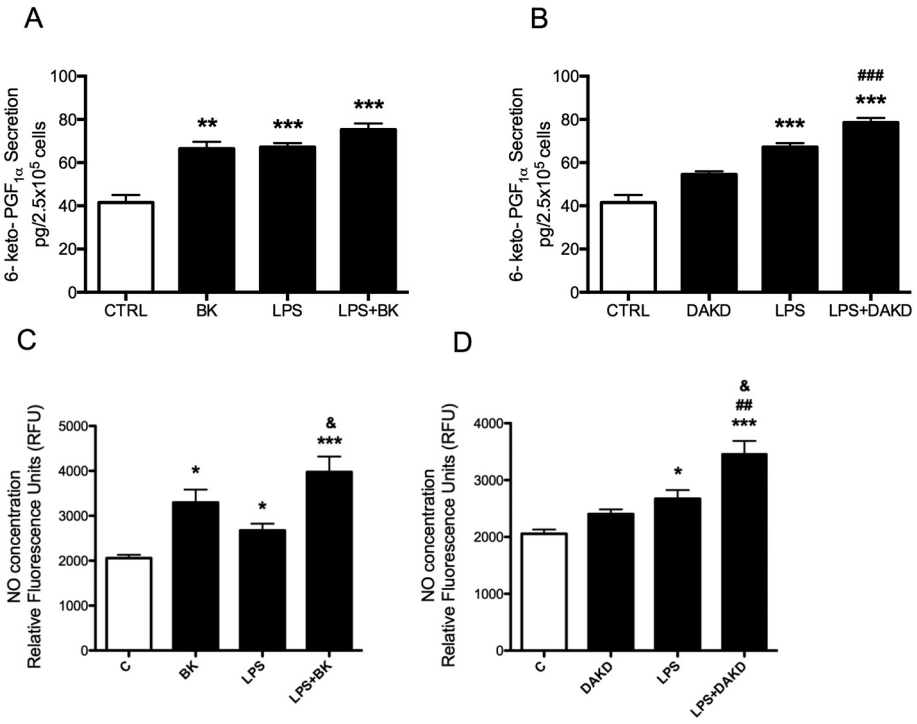


**Fig. 7.** TLR4 pre-activation enhances kinin effects on $PGI_2$ secretion and NO production in CF. Rat CF were pretreated with 1 μg/mL LPS for 48 h to induce B1R expression. Then, CF were washed and media was replaced and incubated with (A, C) 100 nM BK, 1 μg/mL LPS or LPS + BK for 24 h; or (B, D) 100 nM DAKD, 1 μg/mL LPS or LPS + DAKD for 24 h before measuring $PGI_2$ secretion by ELISA kit assay (A,B) or NO production by immunofluorescence kit assay (C,D). *p < 0.05; **p < 0.01; ***p < 0.001 vs C. ##p < 0.01, ###p < 0.001 vs DAKD. &p < 0.05 vs LPS. Data are mean ± SEM of 4 independent experiments.

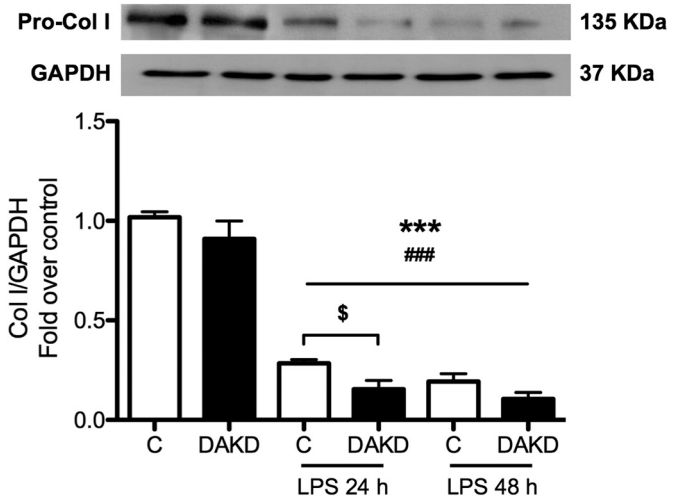


**Fig. 8.** TLR4 pre-activation enhances DAKD effect on collagen I reduction in CF. Rat CF were pretreated with 1 μg/mL LPS for 24 and 48 h to induce B1R expression. Then, culture media was changed and replaced with fresh medium, and after 1 h cells were incubated with 100 nM DAKD for 48 h. Pro-collagen I protein levels were measured by WB with GAPDH as loading control. ***p < 0.001 vs C (without LPS). ###p < 0.001 vs DAKD (without LPS). $p < 0.05 vs C (LPS 24). Data are mean ± SEM of 5 independent experiments.

this, we showed that LPS induced TNF-α expression in CF (Humeres et al., 2016), and we also showed that TNF-α was capable of increasing B1R protein levels in rat CF, but only after 72 h of stimulation (Supplementary Fig. 1). In LPS-treated CF, an increase in pro-IL-1β expression was observed, but not its secretion due to pro-IL-1β needing ATP to assemble and activate the NRLP3 inflammasome which is necessary to cleave pro-IL-1β and secrete IL-1β active form to the culture media (Boza et al., 2016). Collectively, with these results we can postulate that TLR4 and IL1R1 are participating in B1R expression, through an alternative or non-classical pathway.

### 4.2. TLR4 activation increases B1R and decreases α-SMA protein expression in cardiac fibroblasts

Our next step was to show whether LPS increased B1R expression and function. There are no previous reports to establish a direct relationship between TLR4 activation and B1R expression in CF and CMF. Previously, we demonstrated TLR4 presence in CF and CMF (Boza et al., 2016). On the other hand, B1R was poorly expressed in CF and highly expressed in CMF, mainly through a TGF-β1-dependent mechanism (Catalán et al., 2012). Our results showed that LPS through TLR4 activation increased B1R protein levels in a time-dependent manner. Accordingly, TLR4 activation increased B1R mRNA and protein levels in rabbit skin fibroblasts and smooth muscle cells (Bawolak et al., 2008). However, despite these favorable results, in CMF B1R protein levels were decreased. Certainly, molecular mechanisms of mRNA degradation could alter protein expression levels. Nevertheless, our results show that protein expression of the B1R is increased within 24 h and remains relatively stable for up to 72 h. We have previously shown in CF that TGF-β1, by a mechanism involving Smad protein activation, induced B1R expression which is linked to CF-to-CMF differentiation (Catalán et al., 2012). Moreover, we also recently reported that in CF LPS prevented CF-to-CMF differentiation, and also reversed the CMF phenotype induced by TGF-β1 by decreasing Smad3 phosphorylation and increasing Smad7 protein levels (Bolívar et al., 2017) supporting B1R decreased protein levels in CMF. Thus B1R decrease triggered by LPS may also be part of the dedifferentiation mechanism. Collectively, we found opposite effects of LPS on B1R expression in CF and CMF, and these results suggest that in CF LPS increases B1R protein expression which is in accord with a proinflammatory role of CF, meanwhile, a decrease in B1R protein expression was observed in CMF, which is in accord with an anti-inflammatory and profibrotic role of CMF.

With respect to cellular localization of B1R on CF, Catalán et al., described that it is localized to intracellular vesicles. However, these authors also showed that DAKD pretreatment induced B1R relocation to the membrane (Catalán et al., 2012). Our immunocytochemistry results showed that LPS increased the green fluorescence after 48 h, which is in accordance with our western blot results. However, it has been shown that TGF-β1 also increases B1R protein expression in an autocrine

manner. Thus to discount an autocrine TGF-β1 effect, we treated CF with SB431542 (a TGF-R inhibitor) and LPS. Results showed that LPS + SB increased B1R protein levels, corroborating that TLR4 is key in B1R expression and that it is TGF-β1 independent. Contrary to DAKD (Catalán et al., 2012), LPS did not induce its relocation to the cell membrane, by contrast B1R remained mainly in the nucleus and cytosol with very little in the membrane.

Our results showed that LPS treatment markedly reduced α-SMA protein levels compared to control. A reduction in α-SMA enhances the anti-fibrotic effects of LPS, and these results are in accordance with our previous findings (Bolívar et al., 2017). In this sense, it was reported that α-SMA reduction occurs at the transcriptional level, due to LPS inhibiting TGF-β control elements, avoiding CarG box activation (α-SMA regulators) (Sandbo et al., 2007; Bitzer et al., 2000). However, in CF we showed that α-SMA reduction was dependent on a reduction of Smad3 phosphorylation and on Smad7 activation (Bolívar et al., 2017). In conclusion, we suggest that the effects induced by TLR4 activation preventing CF-to-CMF differentiation can be considered to be anti-fibrotic.

### 4.3. TLR4 activation increases COX-2 and iNOS expression in cardiac fibroblasts

COX-2 and iNOS are two key enzymes involving in signaling pathways activated by B1R. PAMPs are strong COX-2 expression inducers (Kirkby et al., 2013). In this sense, in macrophages, TLR4 activation by LPS induced synthesis and release of $PGI_2$ (a metabolite of COX-2 activation) in a concentration-dependent manner (Park et al., 2007). We have shown that CMF express higher COX-2 expression levels than CF (Catalán et al., 2012), being concordant with results obtained in the present work. In CF and CMF, LPS increased COX-2 levels in a time-dependent manner, and this increase was prevented by TAK-242. These results are interesting because COX-2 activation increases $PGI_2$ production, and we showed that in CF or CMF, LPS-treated $PGI_2$ secretion was increased. $PGI_2$ acts as a negative regulator of collagen (Gallagher et al., 1998), thus we can suggest that TLR4 activation shows an anti-fibrotic role as a consequence of the increase in COX-2 activation. In cardiac and renal tissue $PGI_2$ has been described to play a role in organ homeostasis, and the absence of COXs would generate fibrosis (Nasrallah and Herbert, 2005). In skin cell lines, it has been described that $PGI_2$ analogues were able to suppress fibrotic processes through collagen I reduction (Stratton et al., 2002). Another study described that the use of COX-2 inhibitors was deleterious to the cardiovascular system, mainly by the COX-2 decoupling from $PGI_2$ synthase, the enzyme responsible for synthesis of $PGI_2$ (Ruan et al., 2001).

Another target in the bradykinin signaling pathway is NO which has vasodilator and anti-fibrotic effects. NO is synthesized by nitric oxide synthase (NOS). We previously demonstrated that CF poorly express iNOS (the main NOS subtype implicated in NO production in CF); meanwhile, CMF did not express iNOS (Catalán et al., 2012). This enzyme is inducible by cytokines or other proinflammatory agents like LPS, in almost all cell types; moreover, it is an important participant in the inflammatory process (Förstermann and Sessa, 2012). Our results are quite clear; LPS is a potent inducer of iNOS in CF and more remarkable in CMF, which is an important and novel finding. Therefore, we suggest that the ability of LPS to increase iNOS expression in CMF could be also part of this dedifferentiation process.

### 4.4. TLR4 activation potentiates DAKD effects on PGI2 and NO secretion levels in cardiac fibroblasts

The rationale behind the experiments is that LPS induces B1R expression which results in a higher response to DAKD. We found that TLR4 pre-activation potentiates the BK effects on B2R receptors, evidenced by NO production in LPS and BK treated CF, which was also observed between TLR4 and B1R receptors (evidenced by higher PGI2 and NO secretion) in LPS and DAKD treated CF. Moreover, we noted that DAKD alone did not increase $PGI_2$ or NO production, which is in accordance with previous findings showing that CF do not express active B1R (Catalán et al., 2012). In this regard, there are no data in the literature showing additive or synergic effects between both stimuli on NO and $PGI_2$ production; however, other authors had shown that BK up-regulates the expression of TLR4 and promotes an additive increase in inflammatory responses triggered by LPS (Gutiérrez-Venegas et al., 2012). All together with these antecedents we can suggest an additive or potentiation relationship exists between TLR4 and B1R proinflammatory effects.

### 4.5. TLR4 activation potentiates DAKD effects on collagen reduction in cardiac fibroblasts

LPS treatment reduced collagen I protein levels markedly compared to control and DAKD alone. Moreover, a potentiation effect between TLR4 activation and B1R activation on collagen I secretion was observed. Our previous findings had shown that in CF to activate B1R, 1 pulse of DAKD was needed to relocate B1R in the membrane from perinuclear vesicles, while a second pulse was necessary to decrease collagen I protein levels (Catalán et al., 2012). Our present results show that a single pulse of DAKD did not reduce collagen I; however, in LPS-treated CF a single DAKD pulse is enough to trigger a significant decrease in collagen I protein levels. In this respect, it has been shown that the signaling pathway activated by kinins would allow the release of prostacyclin and by the interaction in an autocrine manner with its receptor IPR, will trigger a signaling cascade to decrease collagen I levels. These results are consistent with our present data showing that LPS + DAKD increases $PGI_2$ secretion levels in an additive manner, which ultimately results in a decrease in collagen I levels.

LPS treatment reduced collagen I protein levels markedly compared to control and DAKD alone. The role of TLR4 in cardiac tissue on collagen I levels remains controversial and unclear. In vivo, the recurrent exposure to subclinical concentrations of LPS produces cardiac fibrosis in mice (Lew et al., 2013). These results are associated with an inflammatory process in which strong immune cell participation leads to cardiac inflammation and collagen deposition. These results appear to contradict our results shown here; however, it is difficult to draw direct comparisons because our work was performed in vitro in isolated cell cultures, without immune cell participation in which the collagen I decrease is a consequence of LPS antagonizing autocrine TGF-β signaling pathways, as described before for α-SMA results, priming an anti-fibrotic effect (Catalán et al., 2012; Sandbo et al., 2007; Bitzer et al., 2000).

Finally, our data are summarized in Fig. 9, and collectively suggest that TLR4 activation induces B1R expression and enhances the DAKD anti-fibrotic effect via B1R activation decreasing collagen I levels. This is due to an increase in COX-2 and iNOS expression levels, having an additional effect between LPS and DAKD on $PGI_2$ secretion and NO production in CF leading to a major decrease in collagen deposition. Therefore, reduction in collagen type I is a desirable effect only if it prevents/remedies fibrosis; however, reduction of collagen synthesis in normal heart adversely affects the integrity of extracellular matrix, hence cardiac function.

## 5. Projections

The activation of TLR4 and B1R by their respective agonists could be relevant to initiate and modulate cardiac inflammation, which is a necessary step for wound healing; however, in a parallel manner both stimuli decrease collagen deposition levels and thus they could prevent cardiac fibrosis development. Most pathological conditions in cardiac tissues involve inflammatory processes, and the response triggered is necessary for adequate tissue healing. Moreover, in cardiac tissue after an injury, the acute inflammatory response should not be avoided,

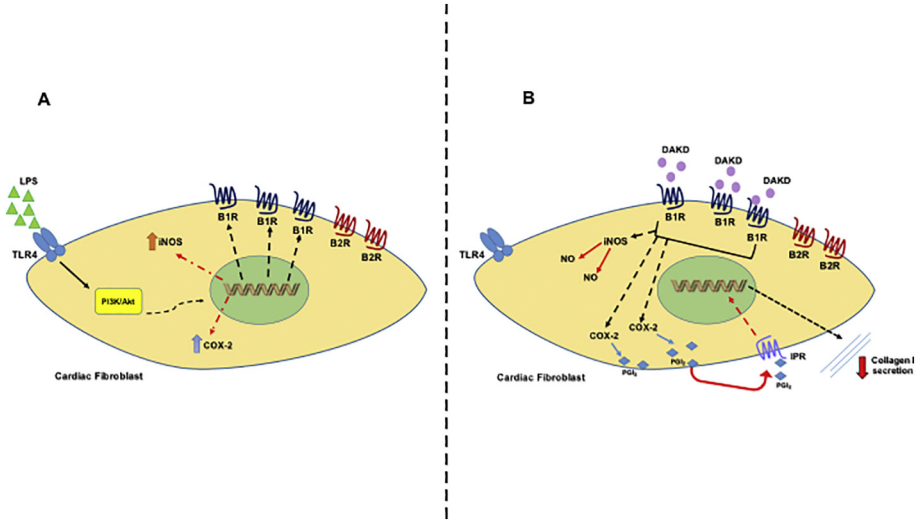


**Fig. 9.** Schematic picture summarizing our findings. A) Cardiac fibroblast express lower B1R, iNOs and COX2 expression levels and LPS treatment through TLR4/PI3K signaling pathway increases B1R, COX and iNOS expression levels. B) In cardiac fibroblast LPS-pretreated, DAKD treatment enhances NO and PGI2 which triggers in an additional manner collagen I reduction.

stopped or mitigated while healing occurs. However, chronic inflammation can lead to cardiac fibrosis. CF and immune system cells contribute to tissue repair in part by secreting cytokines, growth factors, and metalloproteases. We have carried out studies of co-cultivation of cardiac fibroblasts with PBMC, monocytes and/or neutrophils. The results indicate that when there is a physical contact and interaction between both cell types, they are able to determine the phenotype of the other. In this way, cardiac fibroblasts can direct the phenotype change of monocytes to M1 or M2 macrophages depending on whether a proinflammatory or profibrotic stimulus is received by the fibroblasts (Humeres et al., 2016). On the other hand, in later stages, monocytes can change the phenotype of fibroblasts to myofibroblasts, which in some way would be important for the healing process (Olivares-Silva et al., 2018). Therefore, avoiding the fibrotic response without affecting the inflammatory response would be a key point once healing has been established. Certainly, after the healing process has begun, the development of cardiac fibrosis should be preventable; and at a later stage, perhaps regression of fibrosis could also be promoted.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2018.05.011.

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The following is the supplementary data related to this article.

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