

Effects of Atrial Natriuretic Peptide on Rat Ventricular Fibroblasts During Differentiation Into Myofibroblasts

M. MOUBARAK^{1,2}, C. MAGAUD², Y. SALIBA¹, A. CHATELIER², P. BOIS², J.-F. FAIVRE^{2*}, N. FARÈS^{1*}

* These authors contributed equally to this work.

¹Laboratoire de Recherche en Physiologie et Physiopathologie, Pôle Technologie Santé, Faculté de Médecine, Université Saint Joseph, Beyrouth, Liban, ²ERL CNRS 7368, Université de Poitiers, Poitiers, France

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Summary

Atrial natriuretic peptide antifibrotic properties are mainly described in cardiac myocytes or in induced cardiac myofibroblasts (Angiotensin II or TGF- β induced differentiation). In the present work, we investigate the effects of ANP/NPRA/cGMP system in modulating rat cardiac fibroblasts function. Cardiac fibroblasts were isolated from adult Wistar male rats and cultured in the presence of serum in order to induce fibroblasts differentiation. Cultures were then treated with ANP (1 μ M), 8-Br-cGMP (100 μ M) or IBMX (100 μ M), a non-specific phosphodiesterases inhibitor. ANP significantly decreased proliferation rate and collagen secretion. Its effect was mimicked by the cGMP analog, while combining ANP with 8-Br-cGMP did not lead to additional effects. Moreover intracellular cGMP levels were elevated when cells were incubated with ANP confirming that ANP intracellular pathway is mediated by cGMP. Additionally, immunoblotting and immunofluorescence were used to confirm the presence of guanylyl cyclase specific natriuretic peptide receptors A and B. Finally we scanned specific cGMP dependent PDEs *via* RT-qPCR, and noticed that inhibiting all PDEs led to an important decrease in proliferation rate. Effect of ANP became more prominent after 10 culture days, confirming the importance of ANP in fibroblasts to myofibroblasts differentiation. Uncovering cellular aspects of ANP/NPRA/cGMP signaling system provided more elements to help understand cardiac fibrotic process.

Key words

ANP • Fibroblast differentiation • Myofibroblast • cGMP • Cardiac fibrosis

Corresponding author

N. Farès, Laboratoire de Recherche en Physiologie et Physiopathologie, Pôle Technologie Santé, Faculté de Médecine, Université Saint Joseph, rue de Damas, B.P. 11-5076, Riad el Solh, Beyrouth 1107 2180, Liban. Fax: + 961 1 421 023. E-mail: nassim.fares@usj.edu.lb

Introduction

Cardiac fibroblasts (CF) are the most abundant cell type in the adult mammalian heart (Camelliti *et al.* 2005). They play a critical role in normal myocardial structure and function (Manabe *et al.* 2002, Benamer *et al.* 2009). Under pathological conditions, cardiac fibroblasts gain the ability to differentiate into myofibroblasts (MyoFb). While the principal role of myofibroblasts is to repair tissue injury, their persistent activation results in a maladaptive response, leading to cardiac fibrosis and consequently cardiac dysfunction (Kong *et al.* 2013). MyoFb are characterized by increased expression of many extracellular matrix (ECM) markers such as alpha smooth muscle actin (α -SMA), collagen, fibronectin, and fibroblast growth factor-2 (Banerjee *et al.* 2006, Souders *et al.* 2009). They also express high levels of pro-inflammatory molecules and matrix metalloproteinases (Santiago *et al.* 2010). Since collagen is a major determinant of myocardial structural integrity, its disproportionate increase in the heart leads to cardiac fibrosis (van Nieuwenhoven and Turner 2013).

Parthasarathy *et al.* (2013) reported that atrial natriuretic peptide (ANP) decreased both collagen secretion and synthesis.

Atrial natriuretic peptide is a cardiac hormone primarily stored within atrial granules. It reaches the circulation in response to cardiac volume overload inducing vasodilation and enhancing natriuresis, diuresis, and plasma shift (Rubattu *et al.* 2008, Arjamaa 2014). At the molecular and cellular levels, the effects of ANP are primarily mediated through the guanylyl cyclase (GC) activity of natriuretic peptide receptors NPRs (especially NPR-A) and the releasing of cyclic GMP (cGMP) (Potter *et al.* 2006). cGMP is involved in a myriad of cellular functions and is produced by two main types of GCs that differ in their cellular localization and specific ligands activation (Tsai and Kass 2009, Francis *et al.* 2010). While plasma membrane-bound GC is activated by natriuretic peptides, cytosolic-soluble GC is activated by nitric oxide (Fischmeister *et al.* 2005). Phosphodiesterase (PDE) subtypes regulate distinct cellular functions by selectively degrading different cAMP and cGMP pools (Levy 2013, Lu *et al.* 2013); therefore they play crucial roles in regulating the amplitude and duration of cyclic nucleotide signaling (Qvigstad *et al.* 2010). Whereas some of these studies have exposed the involvement of ANP/NPR-A signaling system in the regulation of ECM collagen remodeling in cardiac fibroblasts, the downstream of signaling pathways regulating collagen production during the differentiation process of CF to MyoFb is yet unclear, and the cellular mechanisms underlying natriuretic peptide cGMP-dependent receptor activity are not well understood.

The aim of our study was to further investigate the ANP/NPR-A/cGMP system in serum-stimulated cultured adult rat cardiac myofibroblasts, for better understanding of cardiac fibrogenesis signaling process.

Materials and Methods

Animals

Wistar Strain male albino rats weighing 200-300 g were procured from the "Centre d'Elevage R. Janvier" (Le Genest-Saint Isle, France). The animals were housed in cages under proper environmental conditions, were fed ordinary rat chow and had free access to tap water. The experiments were conducted according to the Guiding Principles in the Care and Use of Animals approved by the Council of the American Physiological Society and were in adherence to the Guide for the Care

and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication no. 85-23, revised 1996) and according to the European Parliament Directive 2010/63/EU.

Isolation and culture of adult rat ventricular fibroblasts

Cardiac fibroblasts were isolated from adult rat and maintained in primary culture as previously described (Benamer *et al.* 2009). Briefly, rats were anesthetized with Ketamine (RotexMedica, 75 mg/kg) and Xylazine (Interchemie, 10 mg/kg). The heart was quickly removed and transferred to Tyrode's solution, containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1.8 MgCl₂, 10 HEPES, 10 glucose, pH 7.4. Ventricles were isolated and dissected in Ca²⁺-free Krebs solution containing (in mM): 35 NaCl, 7.75 KCl, 20 Na₂HPO₄, 25 NaHCO₃, 1.18 KH₂PO₄, 10 HEPES, 10 Glucose, 70 Sucrose pH 7.4, supplemented with 30 mM 2,3-butanedione 2-monoxime (BDM) and 0.5 mM EGTA. Ventricles were digested by two successive enzymatic digestion steps. The first pre-digestion bath (15 min) contained collagenase type V (165.1 U/ml) and protease type XXIV (4.62 U/ml) (Sigma-Aldrich) while the second bath (10 min) contained only collagenase (157 U/ml). The remaining tissue parts and cells were then recovered, and a step of gentle stirring was applied in a modified Tyrode's solution (in mM): 130 NaCl, 4.8 KCl, 1.2 KH₂PO₄, 25 HEPES, 5 glucose, pH 7.4, supplemented with 2% BSA and 0.15 mM EGTA. Cardiomyocytes were discarded after the first centrifugation (500 rpm, 10 min), then fibroblasts collected after the second one (2000 rpm, 10 min) were resuspended in DMEM (Biowhittaker) containing 10% FBS (Lonza), 1% penicillin/streptomycin and 1% insulin. The culture medium was replaced with fresh medium every 2 days. This method resulted in optimized fibroblast cell cultures that were not contaminated by endothelial and/or smooth muscle cells as controlled by immunolabeling studies reported in a previous study (Benamer *et al.* 2009).

Chemicals

Atrial natriuretic peptide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP), 3-isobutyl-1-methylxanthine (IBMX) and the monoclonal β -actin antibody (A2228) were purchased from Sigma Chemical Co. Rat specific polyclonal natriuretic peptide receptor A (ab14356) and B (ab14357)

antibodies were purchased from Abcam.

MTT cell proliferation assay

This technique is based on the transformation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to an insoluble formazan salt by active mitochondria (Mosmann 1983). Ventricular fibroblasts were cultured in 96-well plates (seeded at 20000 cells per well) and grown for 12 days in medium alone or in medium supplemented with different chemicals: ANP, 8-Br cGMP and IBMX+ANP. The test was applied every 2 days from day 4 until day 12. A concentration of 0.5 mg/ml of MTT was added to each well and the plates were incubated for 4 h at 37 °C. Formazan crystals were then dissolved in dimethyl sulfoxide (100 µl/well). Formazan was quantified on a spectrophotometer at 570 nm (in triplicate).

Enzyme-linked immunosorbent assay (ELISA) for cGMP

Cells were cultured in 6-well plates (seeded at 800 000 cells/well) for 12 days with different treatments: ANP, 8-Br-cGMP and IBMX+ANP. The medium was aspirated, and 1 ml of cold PBS was added to each well and the plate was frozen at -80 °C. The plates were then unfrozen on ice, the cells were scraped and mixed with 1 volume of cold HCl (300 µl/well) by vortex. After 5 min of incubation at room temperature, lysate was centrifuged at 4500 rpm for 15 min and the pellet was removed. The supernatant was used to assess the level of cGMP which was measured after acetylation using the cyclic GMP ELISA kit (Abcam).

Collagen measurements by SIRCOL collagen kit assay

The SIRCOL collagen assay (Biocolor) was used to evaluate total collagen quantity in cell culture medium supernatants. Sirius Red (1 ml), an anionic dye that reacts specifically with (Gly-X-Y)_n tripeptide in the triple-helix sequence of mammalian collagens under assay conditions, was added to 100 µl of supernatant and incubated under gentle rotation for 30 min at room temperature. After centrifugation for 10 min at 13 000 rpm, the collagen-bound dye was dissolved with 1 ml of 0.5 M NaOH, and the absorbance was measured at 555 nm (in duplicate).

Western blotting

Fibroblasts cultured in 6 well plates (seeded at 1 million cells/well) were washed with ice-cold PBS and lysed by scraping the cells into 100 µl of lysis buffer (in

mmol/l: Tris-HCl 50, NaCl 150, EDTA 5, 0.05 % Igepal, 1 % deoxycholic acid, 1 % Triton X-100, 0.1 % SDS) containing protease inhibitors (Protease Inhibitor Cocktail, Sigma). Cell lysates were then incubated for 30 min on ice, sonicated then centrifuged at 14 000 rpm for 10 min at 4 °C. Protein quantities were measured using DC protein assay (Biorad) with BSA as a reference. Soluble cell lysates were denatured for 1 h at 37 °C in Laemmli 2X buffer (in mmol/l) Tris-HCl 126, SDS 4 %, glycerol 20 %, bromophenol blue 0.02 %, beta-mercaptoethanol 5 %. Protein samples (50 µg) obtained from the myofibroblasts, were separated using 8 % SDS-PAGE and then transferred to nitrocellulose membranes (Bio-Rad apparatus). Membranes were blocked for 1 h in TBS-Tween blocking solution (in mmol/l: Tris-HCl 100, NaCl 150 and Tween-20 0.1 %) with 5 % BSA and then probed overnight with gentle agitation at 4 °C with primary antibodies (1:5000 for NPRs and 1:10000 for beta-actin). Membranes were then washed three times for 5 min incubated for 1 h at room temperature with specific anti-rabbit or anti-goat horseradish peroxidase-conjugated secondary antibodies (1:10000, Interchim). Membranes were revealed with ECL chemiluminescent substrate (Amersham). Signal intensities of bands in the immunoblots were quantified using ImageJ analysis software.

Immunofluorescence staining

Fibroblasts were cultured on coverslips and grown in DMEM for immunofluorescence (IF). Cells were fixed with paraformaldehyde (PFA) for 10 min, permeabilized by a 10 min incubation in PBS containing 0.5 % Triton X-100, and blocked in PBS containing 5 % BSA. Samples were then incubated overnight at 4 °C with primary antibodies anti-NPRA and anti-NPRB (1/100) in PBS containing 1 % BSA and 0.1 % Triton X-100, after which they were incubated for 1 h at room temperature with the fluorophore-conjugated secondary antibody Alexa Fluor 555 in the same solution (1/200) and with TOPRO 3 (diluted 1/1000) to label cell nuclei. Coverslips were rinsed and mounted using fluorescence mounting medium (Vectashield, Vector Laboratories) on glass microscope slides and examined by confocal microscopy.

Data analysis and statistics

Results are presented as mean ± SEM. Statistical analysis was performed with Student t-test or one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* tests for multiple comparisons using Origin 8.0

(Microcal Software, Inc.). The results are expressed as mean \pm SEM and a p value of less than 0.05 was considered as statistically significant.

Results

Functional effects of ANP on cell proliferation and collagen secretion

Fibroblasts are known to be involved in cardiac remodeling process, notably by their ability to proliferate, to differentiate into myofibroblasts and to secrete extracellular matrix proteins, including collagen. We have reported previously that fibroblasts are able to differentiate spontaneously into myofibroblasts after 6 to 8 days in culture. In the present study, we evaluated the effect of ANP on the properties of cultured fibroblasts. Cells were incubated in either normal medium or in medium supplemented with ANP at a concentration of 1 μ M. Fibroblast cell number was estimated by MTT test at different times following cell isolation, i.e. after 6, 8, 10 and 12 days of culture. As illustrated in Figure 1A, the number of fibroblasts increased with culture duration in control condition, as previously reported (Benamer *et al.* 2009). ANP was able to reduce cell proliferation relative to control from 8 days of culture and this effect became significant from 10 days of culture. In parallel, collagen secretion was measured by Sircol test in the culture medium at the same times and under the same experimental conditions. Results are illustrated in Figure 1B, and show that ANP significantly reduced collagen secretion from 8 days of culture. These results suggest that ANP decreases cardiac fibroblast proliferation and secretion properties after differentiation into myofibroblasts, confirming antifibrotic effect of natriuretic peptide in our experimental conditions. In another set of experiments, we found that ANP 1 μ M was able to reduce cell proliferation and collagen secretion increase induced by 0.1 μ M Angiotensin II (data not shown).

Natriuretic peptide receptors identification in cultured rat cardiac myofibroblasts

To confirm the presence of the main receptors commonly reported to account for ANP effects, we evaluated expression of guanylyl cyclase type receptors NPRA and NPRB in our cellular model. Western blot experiments were performed after 12 days of culture (n=4). Left panel of Figure 2A illustrates immunoblots showing the expression of both isoforms NPRA and

NPRB. Right panel of Figure 2A depicts average intensity measured for NPRA and NPRB proteins, relative to β -actin on 4 independent experiments. Unspecific binding was checked on HeLa cells, which confirms the absence of NPRA expression on this type of cells. On the other hand, confocal microscopy with separate immunofluorescence staining was performed under permeabilized conditions with NPRA and NPRB antibodies. Cells incubated with these specific antibodies revealed positive staining for both isoforms (Fig. 2B).

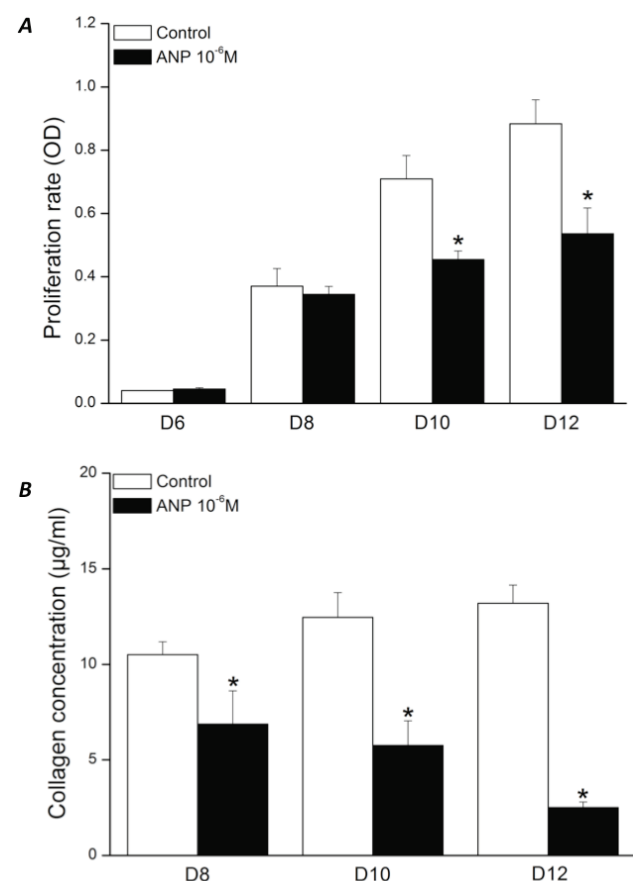


Fig. 1. Effects of ANP on proliferation and collagen secretion in rat cardiac fibroblasts. **(A):** The effects of ANP were evaluated on ventricular fibroblast proliferation by MTT test from 6 to 12 days of culture every 48 h (n=5). **(B):** The secretion of collagen was measured from 8 to 12 days (n=5) in the absence (open bars) and the presence of ANP (10⁻⁶ M) (solid bars). * p<0.05 vs Control

Functional effects of cGMP on cell proliferation and collagen secretion

Because NPRA and NPRB receptors are known to be linked to guanylyl cyclase, we checked whether the effects previously obtained with ANP could be reproduced by cGMP. For this purpose, we investigated the effect of 8-bromo-cGMP, a membrane-permeant and

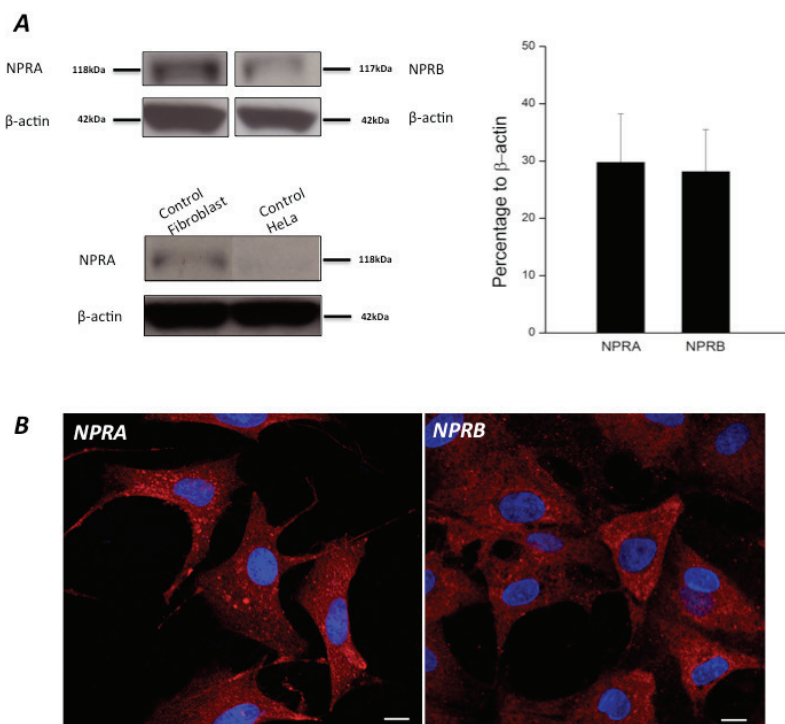


Fig. 2. Expression of type A and type B of natriuretic peptide receptors in cardiac myofibroblasts after 12 days of culture. **(A):** Western blots were performed on protein extracts obtained using NPRA, NPRB and β -actin antibodies. Blotting analysis on right panel illustrates the result of 4 experiments. Non-specific binding was assessed on HeLa cells. **(B):** Positive immunofluorescence staining with NPRA (left panel, red labeling) and NPRB (right panel, red labeling) antibodies on cardiac myofibroblasts. This result is typical for that obtained in 4 independent experiments. Scale bars: 50 μ m

metabolically resistant cGMP analog, on cardiac fibroblasts cultures. Again, cell proliferation and collagen secretion were recorded as markers of fibroblast function. As shown in Figure 3, 8-bromo-cGMP induced similar effect as previously obtained with ANP. Indeed, spontaneous cell proliferation increase was reduced by 8-bromo-cGMP as fibroblasts differentiate into myofibroblasts and this effect reached statistical significance from 10 days of culture. Interestingly, the simultaneous application of 8-bromo-cGMP and ANP did not lead to additional effects (Fig. 3B), suggesting that the intracellular pathway involved in the effects of both ligands is the same. Finally, we found that 8-bromo-cGMP was able to significantly reduce collagen secretion (Fig. 3C), an effect which resembles that previously obtained with ANP.

Phosphodiesterases in rat cardiac myofibroblasts

The results obtained so far suggest that ANP reduces fibroblast function through the activation of NPRA/NPRB receptors which increase intracellular cGMP levels. Our last set of experiments aimed at evaluating the influence of phosphodiesterases (PDEs) involved in cGMP degradation. The chosen phosphodiesterases are the known cGMP-dependent isoforms with the second messenger cGMP acting as a regulator and/or a substrate (Maurice *et al.* 2003, Lugnier 2006, Houslay *et al.* 2007, Omori and Kotera 2007, Miller and Yan 2010, Lee and Kass 2012).

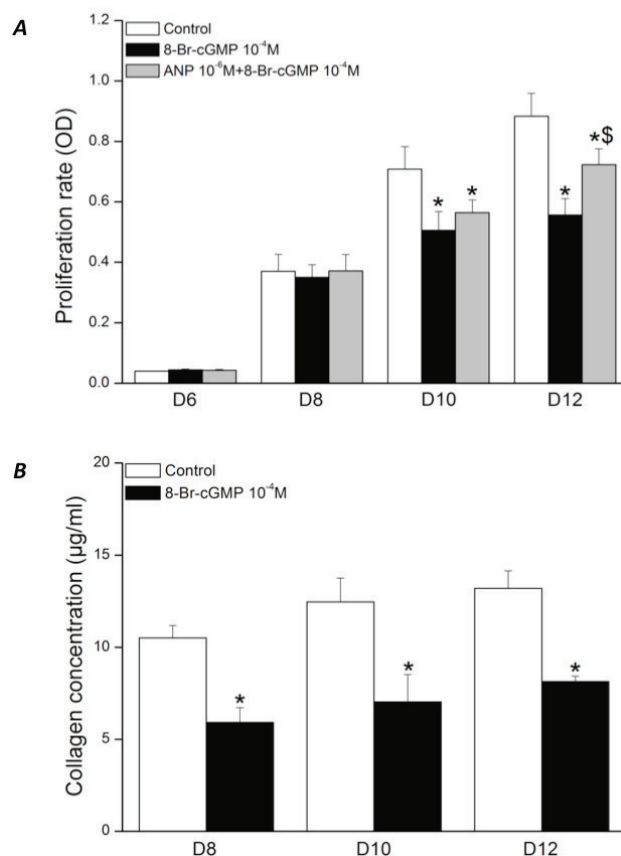


Fig. 3. Effects of 8-Br-cGMP on proliferation and collagen secretion in rat cardiac fibroblasts. The effects of 8-Br-cGMP and ANP plus 8-Br-cGMP **(A)** were evaluated on ventricular fibroblast proliferation by MTT test from 6 to 12 days of culture every 48 h (n=5). **(B):** The secretion of collagen was measured from 8 to 12 days (n=5) in the absence (open bars) and the presence of 8-Br-cGMP (solid bars). * $p < 0.05$ vs Control, \$ $p < 0.05$ vs 8-Br-cGMP

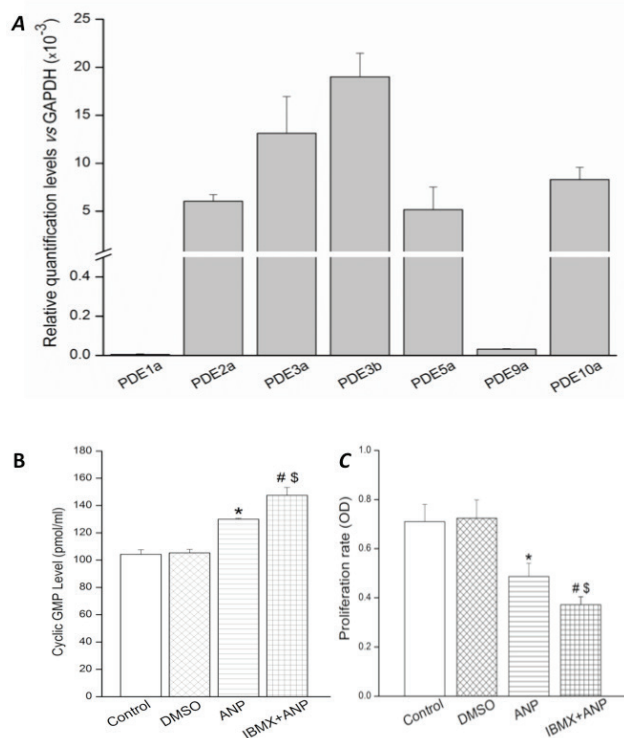


Fig. 4. Relative expression of cGMP-specific phosphodiesterase isoforms and effect of PDE inhibition in cardiac myofibroblasts. All experiments were performed after 12 days of culture. **(A):** Relative expression of cGMP-PDE isoforms evaluated by RT-qPCR using GAPDH as internal control ($n=3$). **(B and C):** Intracellular concentration of cyclic GMP (B, $n=5$) and myofibroblast cell number (C, $n=3$) measured in the absence and in the presence of DMSO (0.1%), ANP (10^{-6} M) and IBMX (10^{-4} M) + ANP (10^{-6} M). * $p < 0.05$ vs Control; # $p < 0.05$ vs DMSO and \$ $p < 0.05$ vs ANP

First, we examined the expression profile of cGMP-PDEs using RT-qPCR in fibroblasts cultured during 12 days. The result illustrated in Figure 4A demonstrates that multiple cGMP-PDEs are expressed in cardiac myofibroblasts and may be involved in cGMP level modulation. In order to correlate ANP effects, PDE activity and cGMP level, intracellular cGMP concentration was measured using enzyme immunoassay in different experimental conditions. As illustrated in Figure 4B, DMSO 0.1% which was used at the same concentration to prepare IBMX solution, did not modify cGMP level. On the opposite, ANP 1 μ M resulted in an expected and significant increase in cGMP levels in comparison to control, strengthening the hypothesis that NPR receptors solicited by ANP are able to modulate intracellular cGMP concentration. When ANP was applied in the presence of IBMX, a potent and non-selective PDE inhibitor, intracellular cGMP concentration was further increased, suggesting that cGMP level is indeed balanced between guanylyl cyclase activity and

PDE-induced degradation. Accordingly, Figure 4C shows that ANP-induced decrease in myofibroblasts cell number relative to control after 12 days of culture was more pronounced in the presence of IBMX.

Discussion

In the present work, we report the effects of cardiac natriuretic peptides, especially ANP, in modulating rat cardiac fibroblasts differentiation. We demonstrate that ANP reduces cell proliferation and collagen secretion as fibroblasts differentiate into myofibroblasts. Both effects are mimicked by an increase of intracellular cGMP, suggesting the involvement of NPRA and/or NPRB receptors, which are equally expressed in cardiac myofibroblasts. Finally, we show that ANP effects are further increased by cGMP-PDE inhibition, strengthening the possible implication of cGMP in fibroblast and myofibroblast physiological regulation.

Our work shows, for the first time, the effects of ANP on cellular proliferation and collagen secretion during differentiation of cultured cardiac fibroblasts into myofibroblasts. ANP's anti-fibrotic effects gain statistical significance after 10 days of culture (1.5 fold decrease in proliferation and fivefold decrease in collagen secretion). These results revealed the importance of ANP effects not only on cardiac fibroblasts (Souders *et al.* 2009, Santiago *et al.* 2010), but also during fibroblasts to myofibroblasts differentiation. The fact that collagen secretion even decreased with culture duration in the presence of ANP whereas cell proliferation still increased suggests that ANP major effect is on differentiation more than on proliferation. ANP has been investigated in many works (Patel *et al.* 2005, Rubattu *et al.* 2008). Its antifibrotic properties are mainly described in cardiomyocytes (Potter 2011, Dickey *et al.* 2012, Volpe *et al.* 2014). The atrial natriuretic peptide and its receptor (NPR) are able to shape the whole underlying signaling system by inducing various physiological effects in a cell specific or tissue specific manner. Studies have shown that ANP inhibited the proliferation of mouse mesangial cell by suppressing the phosphorylation of MAPKs (Tripathi and Pandey 2012) and fetal sheep cardiomyocyte proliferation by inhibiting ERK and AKT phosphorylation (O'Tierney *et al.* 2010). Moreover, our results show ANP effect passes through cGMP signaling according to many previous studies (Fischmeister *et al.* 2005, Castro *et al.* 2010). cGMP, mimicking ANP's effects, reflected an inhibitive

response on cellular proliferation and a decrease in collagen secretion. This result has been demonstrated on collagen secretion levels with a different approach on freshly dissociated CFs (Parthasarathy *et al.* 2013). When combined with 8-Br-cGMP, we noticed that ANP's effects, especially on proliferation were not enhanced. This suggests that once intracellular pathway is saturated by cGMP, it can no further activate the ANP/NPRA system and therefore this system seems to be directly regulated by cGMP in rat cardiac myofibroblasts.

In parallel, the effects of ANP were enhanced and cGMP levels were increased in cardiac myofibroblasts, when IBMX was added to the culture media to non-selectively inhibit PDEs. This resulted in a strong inhibitory outcome on cellular proliferation (twofold decrease). Recent studies have shown that an increase in cGMP and specifically activation of the cGKI (GMP-dependent protein kinase I) inhibits the differentiation of cardiac fibroblasts into myofibroblasts, induced by TGF- β or Angiotensin II (Patrucco *et al.* 2014).

Furthermore, scanning and quantifying cGMP dependent PDE isoenzymes was performed on 12 days serum induced myofibroblasts and reveal 5 different transcript expression. Expression of these specific PDEs seems to have a central role in modulating cGMP (Qvigstad *et al.* 2009, Vettel *et al.* 2014), thus modulating the ANP/NPRA system. Lu *et al.* (2013) quantified PDEs in TGF- β induced cardiac myofibroblasts obtained from Sprague-Dawley rats, and their studies have showed that cAMP can prevent the CFs to myofibroblasts transformation and that increased cAMP levels can reverse the profibrotic myofibroblastic phenotype.

Finally we confirmed the presence of the

guanylyl cyclase NP receptors by immunoblot and confocal microscopy in serum induced cardiac myofibroblasts.

Recently, Bice *et al.* (2014) revealed the importance of cGMP as therapeutic agent in ischemic heart diseases. Moreover, Huntley *et al.* (2006, 2010) exposed the importance of BNP/cGMP interaction in the cardiac repair after myocardial infarction and his potential anti-proliferative effects in human cardiac fibroblasts. Based on these works, it would be interesting to study cGMP modulation in our cellular model, by determining the intracellular changes in cardiac fibrosis process *via* ERK1/2 (Sun *et al.* 2009) or GATA4 (Jankowski 2009, Glen *et al.* 2009) and their position in natriuretic peptides signaling pathway (especially ANP). Furthermore targeting specific PDEs, involved in cGMP homeostasis, can provide important information concerning the precise implicated isoforms in cardiac fibroblasts-myofibroblasts differentiation.

In conclusion, this study showed the important role of ANP/NPRA signaling in fibroblast to myofibroblast differentiation. cGMP and specific PDEs modulating cell proliferation, migration and differentiation provide new insights into better understanding cardiac fibrosis in pathogenic situations.

Conflict of Interest

There is no conflict of interest.

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