Apelin prevents cardiac fibroblast activation and collagen production through inhibition of sphingosine kinase 1

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Aims

Activation of cardiac fibroblasts and their differentiation into myofibroblasts is a key event in the progression of cardiac fibrosis that leads to end-stage heart failure. Apelin, an adipocyte-derived factor, exhibits a number of cardioprotective properties; however, whether apelin is involved in cardiac fibroblast activation and myofibroblast formation remains unknown. The aim of this study was to determine the effects of apelin in activated cardiac fibroblasts, the potential related mechanisms and impact on cardiac fibrotic remodelling process.

Methods and results

In vitro experiments were performed in mouse cardiac fibroblasts obtained from normal and pressure-overload hearts. Pretreatment of naive cardiac fibroblasts with apelin (1–100 nM) inhibited Transforming growth factor-β (TGF-β)-mediated expression of the myofibroblast marker α-smooth muscle actin (α-SMA) and collagen production. Furthermore, apelin decreased the spontaneous collagen production in cardiac fibroblasts isolated from hearts after aortic banding. Knockdown strategy and pharmacological inhibition revealed that prevention of collagen accumulation by apelin was mediated by a reduction in sphingosine kinase 1 (SphK1) activity.

In vivo studies using the aortic banding model indicated that pretreatment with apelin attenuated the development of myocardial fibrotic remodelling and inhibited cardiac SphK1 activity and α-SMA expression. Moreover, administration of apelin 2 weeks after aortic banding prevented cardiac remodelling by inhibiting myocyte hypertrophy, cardiac fibrosis, and ventricular dysfunction.

Conclusion

Our data provide the first evidence that apelin inhibits TGF-β-stimulated activation of cardiac fibroblasts through a SphK1-dependent mechanism. We also demonstrated that the administration of apelin during the phase of reactive fibrosis prevents structural remodelling of the myocardium and ventricular dysfunction. These findings may have important implications for designing future therapies for myocardial performance during fibrotic remodelling, affecting the clinical management of patients with progressive heart failure.

Keywords

Apelin • Cardiac fibroblast • Fibrosis • Myocardial remodelling

Introduction

Fibrotic remodelling of cardiac tissue is a key determinant of the progression of heart failure, which results in an elevated risk of mortality.1 Cardiac fibrosis is characterized by the induction of profibrotic growth factors and activation of cardiac fibroblasts, which play a key role in the development of myocardial remodelling process.2–4 Activated cardiac fibroblasts change their phenotype and differentiate into myofibroblasts, characterized by expression of α-smooth muscle actin (α-SMA) and production of extracellular...
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matrix (ECM) proteins. Cardiac myofibroblasts contribute to the structural and functional changes in the heart in increased deposition of ECM components, predominantly collagen types I and III, within the interstitium, by regulating autocrine/paracrine factors and by replacing of myocytes with fibrotic scar tissue. The conversion of cardiac fibroblasts to myofibroblasts is controlled by a variety of growth factors, cytokines, and mechanical stimuli. Transforming growth factor-β (TGF-β) is a key mediator of cardiac fibroblast activation and differentiation into hypertensive myofibroblasts. Recent studies have demonstrated a potential link between TGF-β-associated fibrosis and a lysophospholipid sphingosine-1-phosphate (S1P) in numerous cell types, including cardiac fibroblasts. In both fibroblasts and myofibroblasts, TGF-β stimulated expression of sphingosine kinase 1 (SphK1), an enzyme that produces S1P, and siRNA against SphK1 inhibited TGF-β-stimulated collagen production. SphK1 activity was reported to mediate TGF-β-stimulated collagen production through S1P2 receptor-induced activation of Rho kinases.

Apelin is a secreted factor of the adipokine family playing a regulatory role in cardiovascular functions. This bioactive peptide is the endogenous ligand of the APJ receptor, and is emerging as an important therapeutic target in heart failure. In preclinical models, apelin causes nitric oxide-dependent vasodilatation, reduces ventricular preload and afterload, and increases cardiac contractility in failing hearts. There is growing evidence that apelin plays a central role in left ventricular remodelling. Although cardiac fibroblasts have been recognized as key mediators of fibrotic myocardial remodelling in the injured and failing heart, the role of apelin in cardiac fibroblast activation and their differentiation into myofibroblasts has not been defined.

The present study provides the first evidence that apelin inhibits TGF-β-mediated fibrotic responses in cardiac fibroblasts and their phenotypic switching to myofibroblasts. We have identified a new mechanism of apelin-mediated suppression of TGF-β signalling that involves transcriptional repression of SphK1 expression. We also demonstrated that the administration of apelin at the phase of reactive fibrosis prevents cardiac remodelling and ventricular dysfunction induced by pressure overload.

Methods

Preparation of cardiac fibroblasts

The first experiment investigated the effects of apelin on TGF-β-induced activation of cardiac fibroblasts. Mouse cardiac fibroblasts were isolated and cultured according to the method described previously by Eghbali. Isolated cells were cultured in Dulbecco modified Eagle medium–nutrient mixture (DMEM/F-12, Invitrogen, France) containing 10% foetal bovine serum (FBS, Gibco, France). The second experiment tested the effect of apelin on activated fibroblasts isolated from mice subjected to pressure overload by constriction of the ascending aorta for 4 weeks or sham operation. Adherent cells were characterized at passage 1 using immunofluorescence microscopy and found to be positive for vimentin but negative for CD31. Fibroblasts up to passage 3 were used in these studies.

Picosirius red staining

Following treatment, cardiac fibroblasts were fixed in methanol, and incubated in the 0.1% Picosirius red staining solution (Sigma-Aldrich, France) as per manufacturer’s instructions. Picosirius red was solubilised in 0.1 N sodium hydroxide and the optical density was read at 540 nm (SERLABO Technologies, France).

Western blot analysis

Western blot analysis was performed on proteins isolated from tissue homogenates and whole cell lysates as described previously. Anti-α-SMA (Ozyme, Saint Quentin en Yveline, France) was used at 1:2000 dilution.

siRNA transfection

SphK1 was targeted using siRNA along with relevant control siRNA as previously described. Knockdown of SphK1 was assessed by enzyme activity assay and qRT–PCR as described below. Optimal knockdown was obtained 72 h post-transfection.

Measurement of phingosine kinase-1 activity

SphK1 activity was performed as described previously and expressed as picomoles of S1P formed in 1 min of total protein.

Histological determination of fibrosis

Paraffin-embedded left ventricles were cut into 4 μm sections, subjected to Masson’s trichrome staining, and the relationship of blue-stained area to the total area of the whole heart section was analysed by an automated computer-assisted image analysis software (Imagene 2000: Biocom, Les Ulis, France). The interstitial fibrosis of cardiac tissue was scored in a blinded manner in six randomly selected areas per sample.

Real-time RT–PCR analysis

Total RNAs were isolated from cultured mouse cardiac fibroblasts using the RNeasy mini kit (Qiagen). Total RNAs (300 ng) were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) in the presence of a random hexamers. Real-time quantitative PCR for α-SMA and SphK1 was performed as previously described. The expression of target mRNA was normalized to ala1, tubulin, polr2a, and gnb2l1 mRNA expression.

Animal models

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and was performed in accordance with the recommendations of the French Accreditation of the Laboratory Animal Care (approved by the local Centre National de la Recherche Scientifique ethics committee). In 7-week-old male C57BL6/J mice (Charles River Laboratories, l’Arbresle, France), pressure overload was produced by constriction of the ascending aorta as described previously. Sham-operated mice underwent a similar procedure without ligation of the ascending aorta.

Immunostaining

Cultured cardiac fibroblasts were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.01% Triton X-100. After being blocked in 1% BSA, cells were incubated for 1 h with primary antibody against α-SMA (1:100, NeoMarkers, Fremont, CA, USA) followed by FITC-conjugated secondary antibodies (Molecular Probes, France). The fluorescence was examined and photographed using Leica fluorescence microscope.
Experimental protocols

Animals were randomly divided into four groups: (i) sham vehicle (n = 8), (ii) aortic-banded (AB) vehicle (n = 7), (iii) sham apelin (n = 7), and (iv) aortic-banded apelin (n = 8). Mice received intraperitoneally apelin (0.1 μmol/kg/day) or vehicle (PBS) 15 min before aortic banding (AB) surgery and then every 24 h for 4 weeks or 2 weeks after AB (n = 10) or sham operation (n = 5), and then every 24 h for 2 weeks.

Haemodynamic study

Mice were anaesthetized with an intraperitoneal injection of ketamine (125 mg/kg) and xylazine (10 mg/kg). A high-fidelity Millar Mikro-Tip pressure catheter (SPR-671, ADInstruments Ltd, Oxford, UK) was then inserted into the right carotid artery, taking great care to avoid any bleeding. Blood pressure was recorded for 10 min using a Bridge-Amp (ML221) connected to a PowerLab 8/30 acquisition system (ML870, ADInstruments). Data were analysed using LabChart Pro 7.2 software (ADInstruments).

Echocardiographic studies

Animals were anaesthetized with 2% isoflurane and examined with non-invasive echocardiography (echocardiograph Vivid 7 ultrasound, GE) 4 weeks after AB surgery. Cardiac ventricular dimensions were measured on M-mode images at least five times for the number of animals indicated.

Chemicals and reagents

Apelin-13 (Bachem, H-4566, Merseyside, UK), SphK1 inhibitor compound 5c was from Cayman Chemicals (Ann Arbor, USA). All other chemicals not referenced in the protocols above were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Statistical analysis

Data are expressed as mean ± SEM. Comparison of multiple groups was performed by two-way ANOVA followed by a Bonferroni’s post hoc test for in vivo studies using GraphPad Prism version 4.00 for Windows (GraphPad Software, Inc). All other statistical analyses were performed by one-way ANOVA followed by the Bonferroni post hoc test. Statistical significance was defined as P < 0.05.

Results

Apelin inhibits transforming growth factor-β1-mediated fibrotic response in mouse cardiac fibroblasts

Transforming growth factor-β induces differentiation of cardiac fibroblasts into myofibroblasts that secrete ECM components and contribute to cardiac remodelling. Treatment of primary mouse cardiac fibroblasts with increasing apelin concentrations (1, 10, and 100 nM) induced a dose-dependent decrease in α-SMA protein expression induced by TGF-β. At apelin concentrations of 10–100 nM, α-SMA protein expression was similar to that detected in control cells (Figure 1A). These results were confirmed by immunocytofluorescence analysis, showing a significant decrease in α-SMA immunodetection in TGF-β-stimulated cells following apelin treatment (Figure 1B and C).

In addition to blocking α-SMA expression, apelin dose-dependently inhibited TGF-β-induced extracellular collagen accumulation (Figure 1D and E). Similarly to the decrease in α-SMA level, the maximum inhibition of collagen production was observed at apelin concentrations of 10–100 nM (Figure 1E).

Inhibition of collagen production by apelin was further confirmed in activated cardiac fibroblasts isolated from mice subjected to 4 weeks of AB. Unlike naive cardiac fibroblasts requiring TGF-β stimulation for collagen production, fibroblasts from pressure-overload ventricles spontaneously produced significant amounts of collagen (Figure 1F) over the first 48 h in culture. In these fibroblasts, 10 nM apelin induced a significant reduction in total collagen deposition, as assessed by Picosirius red staining (Figure 1F).

Apelin inhibits TGF-β-induced fibrotic response via SphK1-dependent mechanism

Previous studies showed that SphK1 is involved in TGF-β-mediated ECM deposition in cardiac fibroblasts.9 Based on these results, we investigated whether apelin may interfere in TGF-β-dependent activation of SphK1. As shown in Figure 2, incubation of cardiac fibroblasts with apelin in the absence of TGF-β -inhibited SphK1 activity in a dose- (Figure 2A) and time-dependent (Figure 2B) manners. After 24 h incubation, 100 nM apelin inhibited SphK1 activity by 36% (Figure 2A). Stimulation of cardiac fibroblasts with TGF-β induced a 32% increase in SphK1 activity compared with control (Figure 2C). In the presence of 100 nM apelin, TGF-β-stimulation of SphK1 activity was fully prevented and reached values similar to those observed in cardiac fibroblasts treated with apelin alone.

Quantitative RT-PCR analysis demonstrated that treatment with apelin only partially downregulated the levels of SphK1 mRNA (Figure 2D) in the absence of TGF-β. Stimulation of cardiac fibroblasts with TGF-β induced a significant increase in SphK1 mRNA that was fully prevented by apelin treatment. These results show that apelin inhibits both basal and TGF-β-stimulated SphK1 activity. However, the results of the measure of mRNA expression suggest that the mechanisms of inhibition of SphK1 activity by apelin may differ in unstimulated and TGF-β-stimulated cells. In particular, inhibition in basal conditions may involve post-translational mechanisms.

In order to determine the involvement of SphK1 inhibition by apelin on cardiac fibroblast function, we have knocked down SphK1 expression by siRNA or inhibited its activity pharmacologically using SKI-II. As shown in Figure 3, both SphK1 siRNA (Figure 3A and B) and SKI-II (Figure 3C) led to a significant reduction in the TGF-β-induced stimulation of collagen production indicating that TGF-β-induced SphK1 activation is crucial for fibrotic response. According with these results, we found that apelin did not modify collagen accumulation in cardiac fibroblasts treated with SphK1 siRNA or SKI-II.

Next, we investigated whether AMP-activated protein kinase (AMPK), a known energy sensor regulating cellular metabolism activated by apelin,19 is involved in inhibition of SphK1. A time-course study of AMPK activation demonstrated that apelin induced a time-dependent phosphorylation of AMPK in cardiac fibroblasts. The phosphorylation was observed as early as 5 min.
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Apelin prevents myocardial fibrosis in a mouse model of cardiac remodelling

To determine whether our in vitro findings have physiopathological relevance, we evaluated the effects of apelin on cardiac fibrosis in a mouse model of pressure overload. Mice were treated with apelin or vehicle either prior to surgery or 2 weeks after aortic banding. As shown by Masson’s trichrome staining, after 4 weeks of AB we observed increased interstitial fibrosis in mice treated with vehicle, which was significantly reduced by animal pretreatment with apelin (Figure 5A and B). Consistent with these observations, in AB mice, we found a significant increase in the mRNA expression of fibrosis-associated factors, including collagen III, and TGF-β. Such increase was prevented by apelin administration (Figure 5C and D). In addition, apelin treatment also reduced expression of myocardial IL-6 (Figure 5E) supporting a role for apelin in the inflammatory responses associated with ventricular remodelling.

Western blot analysis revealed an upregulation of α-SMA expression in hearts from vehicle-treated AB mice, which was reversed to control levels by treatment with apelin (Figure 6A). Interestingly, treatment with apelin alone decreased α-SMA
protein expression by 40% when compared with control (Figure 6A). Quantitative RT–PCR analysis of α-SMA mRNA showed that apelin completely prevented α-SMA upregulation induced by AB (Figure 6B).

As shown in Figure 6C, SphK1 activity increased in the left ventricles from mice subjected to AB. Treatment with apelin significantly reduced SphK1 activity that reached values similar to those observed in sham-operated animals (Figure 6C).

In clinical practice treatment of cardiac fibrosis often occurs after initiation of myocardial remodelling, we next investigated whether apelin treatment can prevent cardiac fibrosis and dysfunction after the onset of pressure overload ventricular remodelling. Based on preliminary experiments, we chose 2 weeks after pressure overload as at this time point fibrosis was significantly increased when compared with sham animals. Daily treatment of mice with apelin started 2 weeks after AB and was continued until mice sacrifice at Week 4. As shown in Figure 7A, 2 weeks of pressure overload induced prominent fibrosis, as demonstrated by an increased trichrome stained-fibrotic area in left ventricle. Apelin administration significantly inhibited ventricular fibrosis and expression of collagen III, TGF-β, and IL-6 when compared with vehicle-treated animals (Figure 7A–D). These effects were associated to significant improvement of ventricular remodelling and function. Indeed, apelin treatment from Week 2 to Week 4 after AB resulted in significant attenuation of hypertrophy, as measured by the heart weight to body weight (HW/BW) ratio (Figure 7E). These results were confirmed by M-mode echocardiography demonstrating a reduction in left ventricular posterior wall dimensions (LVPWD), interventricular septal dimension (IVST), left ventricular internal dimension diastole and systole (LVIDd and LVIDs, respectively) and the increase in fractional shortening (% FS) in apelin-treated AB mice when compared with vehicle-treated animals (Figure 7F–K). The effects of apelin on ventricular remodelling were independent on modification of blood pressure. Indeed, AB produced a significant increase in systolic and mean blood pressure that was unaffected by apelin.

![Figure 2](image-url) Apelin modulates cardiac fibroblast sphingosine kinase 1 level. (A) Dose-dependent effect of apelin on sphingosine kinase 1 activity in mouse cardiac fibroblasts. Activity of sphingosine kinase 1 was measured after 6 h of treatment with 1, 10, and 100 nM apelin. (B) Time-dependent changes in sphingosine kinase 1 activity in cardiac fibroblasts treated with 100 nM apelin for 6 h and 24 h. (C) Effect of apelin on sphingosine kinase 1 activity in transforming growth factor-β-stimulated cardiac fibroblasts. Cardiac fibroblasts were stimulated with 10 ng/mL transforming growth factor-β for 24 h with or without 100 nM apelin. (D) Effect of apelin on cardiac fibroblast sphingosine kinase 1 mRNA expression in transforming growth factor-β-stimulated cardiac fibroblasts. Sphingosine kinase 1 mRNA levels were measured using qRT–PCR. Data are the mean ± SEM of three independent experiments. *P < 0.05 vs. control (C); **P < 0.01 vs. control; ***P < 0.001 vs. control; §§§P < 0.001 vs. transforming growth factor-β.
administration. In addition, apelin did not modify diastolic blood pressure in both sham and AB animals (Table 1).

**Discussion**

Activation of cardiac fibroblasts and their differentiation into myofibroblasts is a critical event in the progression of heart failure. The results presented in this study demonstrate for the first time that apelin inhibits TGF-β-induced phenotypic switching to myofibroblasts and fibrotic responses in cardiac fibroblasts. In addition, we identified a novel Sphk1-dependent mechanism by which apelin opposes a TGF-β-mediated activation of cardiac fibroblasts. Indeed, we showed that, in unstimulated and in TGF-β-stimulated cardiac fibroblasts, apelin inhibits SphK1 through AMPK-dependent and AMPK-independent mechanisms, respectively. Finally, we demonstrated that the administration of apelin attenuates established cardiac hypertrophy, fibrosis and prevents cardiac contractile dysfunction induced by pressure overload.

A significant finding of this study is that apelin at nanomolar doses inhibits TGF-β-induced phenotypic switching to myofibroblasts and fibrotic responses in cardiac fibroblasts. Transforming growth factor-β is considered a ‘master switch’ in the induction of fibrosis in many tissues including the heart, and blocking TGF-β signalling is predicted to blunt myocardial fibrosis. Since TGF-β has been shown to stimulate the synthesis of ECM proteins, we have addressed whether apelin affects TGF-β-induced fibroblast activation and ECM accumulation. We found that pretreatment of cardiac fibroblasts with apelin reduces TGF-β-mediated collagen production in a dose-dependent manner. The role of apelin in driving collagen formation was also confirmed by in vivo experiments indicating that chronic treatment of mice with low doses of apelin reduces pressure overload-induced enhancement of α-SMA protein expression. In the light of the role of apelin in deactivation...
of TGF-β-mediated fibroitic remodelling, it is reasonable to speculate that apelin is also essential for preserving cardiac fibroblast function by blocking myofibroblast transformation under pathologic conditions. Further work is required to delineate the exact mechanisms underlying apelin signalling in remodelling process during heart failure.
Figure 6 Chronic treatment with apelin decreases pressure overload-induced α-smooth muscle actin expression and sphingosine kinase 1 activation. (A) Representative western blot for α-smooth muscle actin protein expression (top panel) and densitometric analysis (bottom panel) of cardiac tissue obtained from sham (S) or aortic banding (AB) mice treated with apelin (A) or vehicle as indicated above. Equal loading was confirmed by β-tubuline blotting. (B) Expression of myocardial α-smooth muscle actin mRNA in left ventricles measured by qRT–PCR. (C) Effect of apelin treatment on myocardial activity of sphingosine kinase 1 in sham or AB mice. Data are expressed as mean ± SEM. *p < 0.05 vs. vehicle-treated sham; **p < 0.01 vs. vehicle-treated sham; ***p < 0.001 vs. vehicle-treated sham; §p < 0.05 vs. vehicle-treated AB mice; §§p < 0.01 vs. vehicle-treated AB mice.

Figure 7 Post-treatment with apelin reduces myocardial fibrosis and left ventricular dysfunction induced by pressure overload. (A) Quantitative analysis of the Masson’s trichrome sections of left ventricles from mice subjected to 2 or 4 weeks of aortic banding (AB). Treatment with apelin (0.1 μmol/kg/day i.p.) started at Week 2 after AB or sham operation and followed by collection of left ventricles at Week 4 of AB. (B–D) Expression of myocardial collagen type III (B), transforming growth factor-β (C), and IL-6 (D) mRNA in left ventricles was measured using qRT–PCR. (E) The heart weight (HW) to body weight (BW) ratio in sham control and AB mice treated with vehicle or apelin as indicated above. (F–L) Two-dimensionally guided M-mode echocardiographic measurement of interventricular septal dimension (IVST), left ventricular posterior wall dimensions (LVPW), and left ventricular internal dimensions at both diastole and systole (LVIDd and LVIDs, respectively), left ventricular fractional shortening (FS), and heart rate (HR) in sham control and AB mice treated with vehicle or apelin as indicated above. Data are expressed as mean ± SEM; *p < 0.05 vs. vehicle-treated sham; **p < 0.01 vs. vehicle-treated sham; ***p < 0.001 vs. vehicle-treated sham; §p < 0.05 vs. vehicle-treated AB mice; §§p < 0.01 vs. vehicle-treated AB mice; §§§p < 0.001 vs. vehicle-treated AB mice.
To gain a better understanding of the signalling mechanism responsible for the antifibrotic effect of apelin, we examined SphK1 activation, which had been shown to play a critical role in profibrotic responses in cardiac fibroblasts. We have identified SphK1 as the intracellular signalling intermediate involved in the antifibrotic activity of apelin. Indeed, we showed that treatment of cardiac fibroblasts with apelin inhibits TGF-β-mediated up-regulation of SphK1 mRNA and activity. In addition, we demonstrated that pharmacological or genetic inactivation of SphK1 abolishes TGF-β-mediated collagen accumulation. These data are in agreement with recent findings suggesting that SphK1 activity and expression are required for TGF-β-stimulated collagen production through S1P2 receptor-induced activation of Rho kinases. This hypothesis is supported by a recent finding that mice with high levels of SphK1 developed progressive myocardial degeneration and fibrosis, elevated RhoA and Rac1 activity, stimulation of Smad3 phosphorylation, and increased levels of oxidative stress markers through transactivation of S1P3, a major myocardial S1P receptor subtype that couples to Rho GTPases and transactivates Smad signalling. Recent extensive gene profile analyses suggested a hierarchical model of gene regulation in which initial TGF-β/Smad3 signalling activates a number of secondary regulatory pathways and it appears that the SphK1/S1P pathway may be one of these.

Interestingly, we also found that apelin inhibits SphK1 activity in cardiac fibroblasts in the absence of TGF-β. The mechanisms of SphK1 inhibition by apelin and the functional consequences seem to be different in unstimulated and TGF-β-stimulated cardiac fibroblasts. Indeed, in TGF-β-stimulated cells, we showed that inhibition of SphK1 activity was associated with a decrease in SphK1 expression that was independent of AMPK activation. In contrast, in the absence of TGF-β, we found that apelin treatment did not modify significantly SphK1 expression, but it inhibited SphK1 activity through an AMPK-dependent mechanism. These data provide strong support for the idea that distinct signalling machineries supervise regulation of SphK1 by apelin in cardiac fibroblasts. Considering that AMPK behaves as a master regulator of cellular energy metabolism in various cell types, it is conceivable that the activation of AMPK by apelin may be involved in the regulation of cardiac fibroblast metabolism.

According with the results obtained in vitro, we also found that apelin inhibited ECM accumulation in vivo. Experiments performed in pressure overloaded mice showed that pretreatment with low doses of apelin significantly decreased myocardial fibrosis and downregulated expression of collagen, the most abundant protein in the heart. These data are consistent with previous studies performed in a rat model of right ventricle hypertrophy and in apelin gene-deficient mice. Importantly, in our study, we also demonstrated that apelin treatment can block ongoing fibrosis progression and structural changes in the heart induced by pressure overload. Indeed, apelin treatment started at 2 weeks after AB resulted in a significant decrease in cardiac collagen content and attenuation of cardiac hypertrophy as measured by the HW/BW ratio and thickness of the IVS and LVPW. These effects were accompanied by an improvement of the systolic function after 4 weeks of AB. Interestingly, we found that treatment with apelin also reduced expression of IL-6 when compared with vehicle-treated AB mice, suggesting that apelin may attenuate myocardial cardiac accumulation of pro-inflammatory cytokines during the ongoing reactive cardiac remodelling. These results are in agreement with our data from in vitro experiments (Supplementary material), showing that apelin inhibited TGF-β-dependent stimulation of IL-6 production by isolated cardiac fibroblasts. Taken together, our data suggest that apelin treatment abolished development of cardiac hypertrophy as well as prevented fibrosis progression and cardiac contractile dysfunction.

In conclusion, our data show that apelin behaves as a potent suppressor of cardiac fibroblast activation and profibrotic activity. In addition, we showed that apelin administration before and after the onset of ventricular remodelling decreases cardiac fibrosis and improves ventricular function. These data have important implications for future efforts in designing an efficient therapeutic strategy to control or prevent myocardial fibrosis and could therefore be of particular relevance in the clinical management of patients with progressive heart failure.

**Supplementary material**

Supplementary material is available at *European Heart Journal* online.

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