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Cardiovascular Research 76 (2007) 442–452

Cardiovascular Research

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Atrial cardiomyocyte tachycardia alters cardiac fibroblast function: A novel consideration in atrial remodeling ☆

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Received 19 March 2007; received in revised form 18 July 2007; accepted 20 July 2007
Available online 25 July 2007
Time for primary review 16 days

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Abstract

Objective: Atrial fibrillation (AF) causes tachycardia-induced atrial electrical remodeling, contributing to the progressive nature of the arrhythmia. Ventricular dysfunction due to a rapid response to AF can cause structural remodeling, but whether AF itself directly promotes atrial fibrosis is controversial. This study investigated the hypothesis that rapid atrial cardiomyocyte activation produces factors that influence atrial fibroblast proliferation and secretory functions.
Methods: Cultured canine atrial fibroblasts were treated with medium from rapidly-paced atrial cardiomyocytes, non-paced cardiomyocytes and cardiomyocyte-pacing medium only, and analyzed by [^{3}H]thymidine incorporation, Western blot and real-time RT-PCR.
Results: Rapidly-paced cardiomyocyte-conditioned medium reduced [^{3}H]thymidine uptake compared to non-paced cardiomyocyte-conditioned medium and medium alone (~85%, $P<0.01$). Rapidly-paced cardiomyocyte medium increased αSMA protein (~55%, $p<0.001$), collagen-1 (~85%, $P<0.05$) and fibronectin-1 (~205%, $P<0.05$) mRNA expression vs. controls. The angiotensin-1 receptor blocker valsartan attenuated pacing-induced αSMA changes but did not affect fibroblast proliferation. Suppression of contraction with blebbistatin did not prevent tachypacing-induced changes in [^{3}H]thymidine uptake or αSMA upregulation, pointing to a primary role of electrical over mechanical cardiomyocyte activity. Atrial tissue from 1-week atrial-tachypaced dogs with ventricular rate control similarly showed upregulation of αSMA protein (~40%, $P<0.05$), collagen-1 (~380%, $P<0.01$) and fibronectin-1 (~430%, $P<0.001$) mRNA versus shams.
Conclusions: Rapidly-paced cardiomyocytes release substances that profoundly alter cardiac fibroblast function, inducing an activated myofibroblast phenotype that is reflected by increased ECM-gene expression *in vivo*. These findings are consistent with recent observations that AF *per se* may cause ECM remodeling, and have potentially important consequences for understanding and preventing the mechanisms underlying AF progression.
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Keywords: Fibrosis; Remodeling; Extracellular matrix; Angiotensin

1. Introduction

Atrial fibrillation (AF) changes atrial electrophysiological function, providing a possible explanation for the progressive nature of this arrhythmia. There exists a clearly-established link between atrial extracellular matrix (ECM) remodelling and AF maintenance, likely related to uncoupling of cardiomyocytes by intervening fibrotic deposits [1,2]. Clinical AF is commonly associated with underlying heart disease [3]; however, data about structural remodeling as a potential direct consequence of AF are limited [4,5]. Rapid electrical stimulation of cultured atrial cardiomyocytes produces electrical remodeling, recapitulating the principle phenotypic features of atrial-tachycardia remodeling *in vivo* [6]. It remains unclear whether rapid cardiomyocyte activation

☆ Supported by the Canadian Institutes of Health Research.
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doi:10.1016/j.cardiores.2007.07.013

Table 1
Gene-specific primers and TaqMan probe sequences used in real-time RT-PCR analysis

Gene	Forward primer sequence	Reverse primer sequence	Probe sequence	Accession number
Collagen-1 (COL1A1)	CCAAGAGGAGGGCCAAGAA	AGTACCTGAGGCCGTTCTGTA	ACTGGTGGGATGTCTTC	NM_001003090
Fibronectin-1 (FN1)	GTTCGGGAGGAGGTTGTTACC	GAGTCATCTGTAGGCTGGTTTAGG	CCTTGGTCCACAGAGT	XM_536059

influences neighbouring non-cardiomyocyte cells, an issue of particular importance in the context of cardiomyocyte/fibroblast crosstalk during atrial remodeling [7,8]. We tested the hypothesis that tachypaced atrial cardiomyocytes generate factors which influence fibroblast proliferation and ECM-secreting functions. We also sought evidence for consequences of this potential paracrine mechanism in a clinically-relevant experimental model of atrial-tachypacing (ATP)-induced AF [9].

2. Methods

2.1. Cardiomyocyte culture and pacing

HL-1 atrial-derived cardiomyocytes [10] were obtained from William Claycomb (Louisiana State University) and grown to confluence on gelatin-coated culture dishes (Fisher) in culture medium consisting of Complete Claycomb Medium (JRH Biosciences) supplemented with 10%-FBS, 100 μM norepinephrine, 4 mM L-glutamine and 1% penicillin-streptomycin, as previously-described [11]. Confluent HL-1 cells contracted spontaneously at ~0.5 Hz, and were maintained in cardiomyocyte-pacing medium (culture medium with 2% FBS-content) throughout pacing. Cells were subjected to rapid electrical stimulation or cultured in parallel without stimulation. Stimulated cells were paced as previously-described [12] with a C-Pace100-culture pacer and C-Dish100-culture dishes (IonOptix), with 5-ms, 5-Hz square-wave pulses (45±3 V pulse-voltage, ~7 V/cm) for 24 h. Capture efficiency >90% was confirmed by microscopic examination of cell shortening at the beginning and end of stimulation, and pacing resulted in <10% cell death [6]. Conditioned media from rapidly-stimulated myocytes (group 5-Hz), non-paced myocytes (group NP) and cardiomyocyte-pacing medium alone containing no cells (basal, group 0) were transferred to atrial fibroblast cultures isolated from single dogs for parallel study. To exclude non-specific effects of electrical stimulation, experiments were conducted applying electrical stimulation to cardiomyocyte-pacing medium (group 0P). Additional experiments were performed with the excitation–contraction uncoupler (±)-blebbistatin (5 μM, Calbiochem) in the cardiomyocyte-pacing medium throughout electrical stimulation, to evaluate the importance of cardiomyocyte contraction, and with the AT1-receptor blocker, valsartan (10 μM, Novartis), added to the paced myocyte-conditioned medium to assess the role of locally-elaborated angiotensin II. Both blebbistatin and angiotensin were studied in parallel with vehicle-treated controls.

2.2. Fibroblast isolation and culture

Atrial fibroblasts were obtained from right atria of adult mongrel dogs ($n=17$) weighing 23–31 kg, as described previously [13]. The investigation conforms with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996). Only first and second-passage fibroblasts were used, and for all experiments cells from a single dog were plated in culture medium at 200 cells/mm^2. Cells were allowed to adhere, then rendered quiescent by 24 h in serum-free medium, followed by 24-h treatment with conditioned cardiomyocyte medium.

2.3. [^{3}H]Thymidine incorporation assay

Fibroblast proliferation was assayed by [^{3}H]thymidine incorporation as described previously [13]. Fibroblasts were plated in 24-well plates at 50 cells/mm^2 and, following serum starvation, treated for 24 h. Disintegrations per minute (DPM) for each treatment were measured in triplicate.

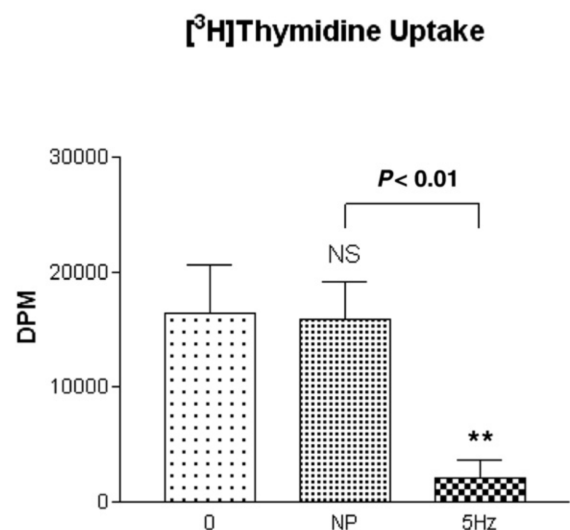

Fig. 1. [^{3}H]Thymidine incorporation (mean±SEM) in atrial fibroblasts treated in parallel with cardiomyocyte-pacing medium alone (basal, 0), non-paced cardiomyocyte-conditioned medium (NP) and rapidly-paced cardiomyocyte-conditioned medium (5Hz); $n=5$, $^{**}p<0.01$ vs. basal. NS=no significant difference vs. basal.

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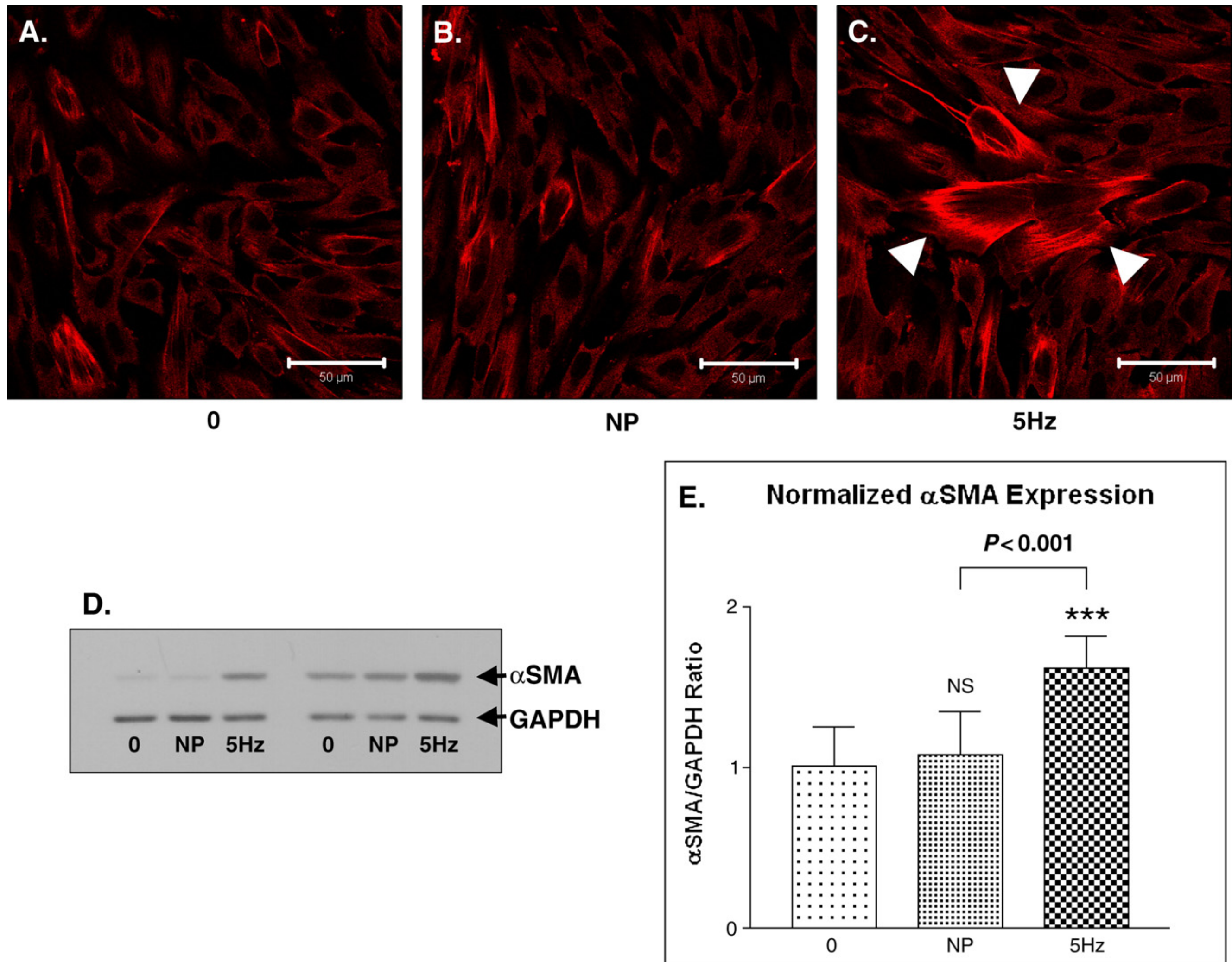

Fig. 2. A–C: Immunofluorescent confocal images with αSMA staining in red from 1 representative plate of treated fibroblasts per group (original magnification × 63, bar = 50 μm, $n=3$). Arrowheads indicate large cells with strong-staining, organized αSMA stress fibers, characteristic of a myofibroblast phenotype. D: Representative αSMA (top) and GAPDH (bottom) bands from two sets of treated fibroblasts. E: Mean ± SEM αSMA-band intensities relative to corresponding GAPDH signals; $n=7$/observation, *** $p<0.001$ vs. basal. NS = no significant difference vs. basal.

2.4. Immunohistochemistry and confocal imaging

Following treatment, fibroblasts plated on glass coverslips in p100 culture dishes were washed with phosphate-buffered saline (PBS) and fixed with 2%-paraformaldehyde. Fixed cells were washed, permeabilized with 0.1% Triton X-100 and blocked with 2%-normal donkey serum (NDS, Jackson ImmunoResearch Laboratories). The cells were then incubated with mouse anti-α-smooth muscle actin (αSMA, Sigma, 1/200) primary antibodies, followed by donkey anti-mouse Alexa Fluor-555 secondary antibodies (Invitrogen, 1/600). Excess antibody was removed by three washes and the coverslip-mounted cells applied to glass slides in Dabco mounting medium (Sigma). Cells were imaged with a Zeiss LSM-510 confocal microscope. Parallel negative-control studies with cells incubated with only secondary antibodies revealed no non-specific staining.

2.5. Ca^{2+}-transients and cell contraction

Intracellular Ca^{2+}-transients and cell shortening were recorded as previously-described [14] with Indo-1 AM microfuorometry and video edge-detection, from 5-Hz paced cultured cardiomyocytes in pacing medium before and after supplementation with (±)-blebbistatin (5 μM, $n=4$).

2.6. Atrial-tachypacing animal model

Adult mongrel dogs ($n=10$) were subjected to 1-week ATP [9,15,16]. Right-atrial pacing was performed at 400 bpm. Ventricular rate was controlled by producing AV-block (radiofrequency-ablation), and a ventricular lead attached to a demand-pacemaker set to 80 bpm. ATP dogs were compared with similarly-instrumented control dogs ($n=8$) with atrial pacemaker inactivated. At sacrifice, right-

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Table 2
GAPDH Western-blot band intensity in arbitrary units*

	Groups	Mean intensity	SEM	*n*
A	0	7506	982	7
	NP	7736	988	7
	5Hz	8078	892	7
B	0	8565	950	4
	0P	8835	751	4
C	0	4822	521	6
	NP	4721	495	6
	5Hz	4651	431	6
	0+Bleb	4548	314	6
	NP+Bleb	5014	334	6
	5Hz+Bleb	5110	171	6
D	0	4110	646	6
	NP	4471	489	6
	5Hz	4356	411	6
	0+Val	4545	296	6
	NP+Val	4747	302	6
	5Hz+Val	4483	257	6
E	CTL	3850	119	7
	ATP	3647	208	7

*There were no statistically-significant differences among groups. Bleb= blebbistatin, Val=valsartan.

atrial free-wall tissue samples were collected, snap-frozen in liquid-N_2 and stored at −80 °C.

2.7. Western-blot analysis

Proteins were extracted from treated fibroblasts and whole right-atrial free-wall tissue as previously-described [13]. Protein was quantified by Bradford assay. Proteins (10 μg/lane) were separated with 10%-polyacrylamide gel-SDS electrophoresis and transferred to PVDF membranes. Membranes were blocked, then incubated with anti-αSMA (1/40,000) and anti-GAPDH (1/5000, RDI) primary antibodies, followed by anti-mouse (1/20,000, Santa Cruz) HRP-conjugated secondary antibodies, and revealed with Western-Lightning chemiluminescence (PerkinElmer). αSMA-band intensities were quantified by video-densitometry and expressed relative to GAPDH-intensity from the same sample.

2.8. RNA extraction and TaqMan real-time RT-PCR

Total RNA was isolated from treated fibroblasts and whole right-atrial free-wall tissue by guanidine thiocyanate–phenol–chloroform extraction [17], then DNase-treated (RNeasy mini kit, Qiagen), quantified and quality-controlled by microelectrophoresis on polyacrylamide gels (Agilent 2100 Bioanalyzer). DNA-contamination was excluded by reverse transcription (RT)-negative polymerase chain reaction (PCR). First-strand cDNA was synthesized from 2-μg total RNA with High Capacity cDNA Archive Kits (Applied Biosystems). Real-time RT-PCR was performed with 6-carboxy-fluorescein (FAM)-labeled fluorogenic collagen-1 and fibronectin-1 TaqMan primers and probes (Assay-by-design, sequences in Table 1) and TaqMan universal master mix (Applied Biosystems) with the Stratagene Mx3000P sequence-detection system [15]. Fluorescence-signals were detected in duplicate, normalized to 18S-ribosomal RNA (Applied Biosystems) and quantified using MxPro QPCR software (Stratagene).

2.9. Statistical analysis

Data are presented as mean±SEM. [^{3}H]Thymidine incorporation, protein and mRNA expression were studied in parallel in different groups and analyzed by repeated-measures analysis of variance (ANOVA). Bonferroni-corrected paired t-tests were applied to evaluate individual mean differences when ANOVA revealed significant group effects. For mRNA and protein expression in whole-tissue samples, a non-paired Student's *t*-test was applied. A two-tailed *P*-value<0.05 was considered statistically-significant.

3. Results

3.1. Conditioned medium-induced fibroblast proliferation

Following 24-h serum starvation, [^{3}H]thymidine uptake was not different between fibroblasts treated with conditioned medium from non-paced myocytes compared to basal (Fig. 1). Conversely, treatment with conditioned medium from rapidly-paced myocytes had a strong anti-proliferative effect, reducing [^{3}H]thymidine uptake by ~85% (n=5, P<0.01).

3.2. Myofibroblast differentiation in response to conditioned medium

Fibroblasts stimulated to differentiate acquire a myofibroblast phenotype, resembling the activated secretory cell type involved in myocardial remodeling and fibrosis, characterized by expression of the contractile protein αSMA [18]. A significantly greater percentage of large cells with strong-staining, organized αSMA stress-fibers were observed in fibroblast cultures treated with rapidly-paced cardiomyocyte medium compared to control groups (Fig. 2A–C). Increased αSMA expression was confirmed quantitatively by Western-

Table 3
18S-ribosomal RNA expression by real-time RT-PCR*

	Groups	Mean Ct	SEM	*n*
A	0	9.78	0.06	7
	NP	9.77	0.02	7
	5Hz	9.91	0.13	7
B	0	9.91	0.09	4
	0P	9.83	0.04	4
C	CTL	11.15	0.37	8
	ATP	11.18	0.14	10

*There were no statistically-significant differences among groups.

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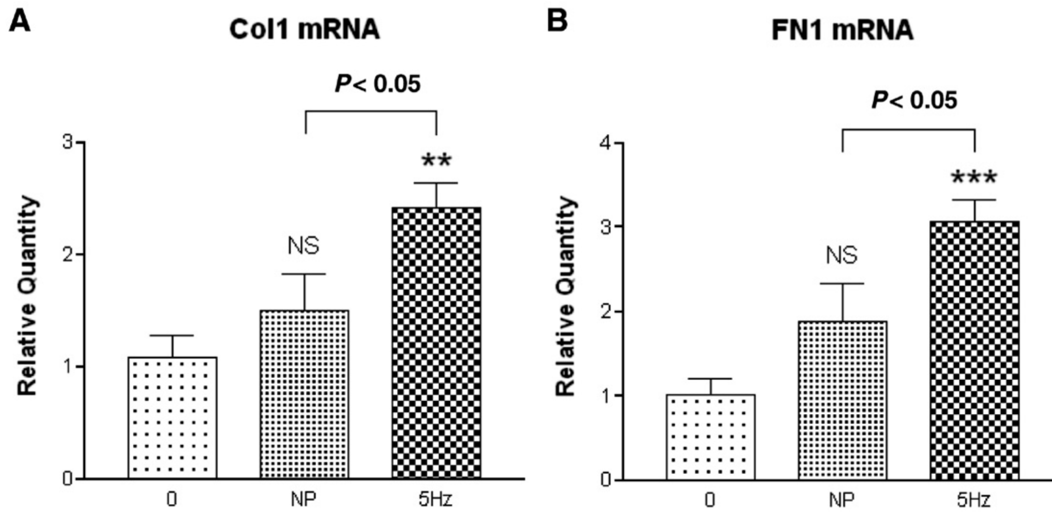

Fig. 3. Mean ± SEM relative quantity of Col1 (A) and FN1 (B) mRNA expression in treated fibroblasts; $n = 7$/observation, ** $p < 0.01$, *** $p < 0.001$ vs. basal. NS = no significant difference vs. basal.

blot analysis. Immunoblots for fibroblasts subjected to each treatment showed distinct bands (Fig. 2D) corresponding to αSMA (~42 kD) and GAPDH (~36 kD). Mean αSMA expression (Fig. 2E) was greater in fibroblasts treated with rapidly-paced cardiomyocyte medium compared to treatment with either non-paced cardiomyocyte medium or pacing

Fig. 4. Control experiments demonstrating no difference in effect of cardiomyocyte-pacing medium alone (basal, 0) versus electrically stimulated cardiomyocyte-pacing medium (0P) on fibroblast function as determined by [^{3}H]Thymidine incorporation (A), αSMA expression (B), or Col1 and FN1 mRNA expression (C); mean ± SEM, $n = 4$/observation.

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medium alone (1.62±0.20 vs. 1.08±0.26 and 1.01±0.24, $n=7$, $p<0.001$). GAPDH expression was unchanged by any treatment (Table 2A).

3.3. ECM-gene expression in treated fibroblasts

We next elected to study gene expression changes in collagen-1 (Col1) and fibronectin-1 (FN1), the two most abundant collagen and non-collagen ECM proteins respectively [19], synthesis and secretion of which are both greatly enhanced in activated myofibroblasts during structural remodeling and fibrosis [20–22]. Treatment of fibroblasts did not alter the expression of the normalizing reference gene, 18S-rRNA (Table 3A). Collagen-1 (Col1, Fig. 3A) and fibronectin-1 (FN1, Fig. 3B) mRNA expression was not different between fibroblasts treated with conditioned medium from non-paced myocytes compared to basal. Collagen-1 (2.41±0.22 vs. 1.49±0.33 and 1.10±0.19) and fibronectin-1 (3.05±0.28 vs. 1.80±0.45 and 1.20±0.19) mRNA expression was significantly increased by conditioned medium from rapidly-paced cardiomyocytes. Taken together with the reduced proliferative rate and increased αSMA expression, results are consistent with the induction of myofibroblast differentiation [23].

3.4. Effects of rapid electrical stimulation of cardiomyocyte-pacing medium

Fibroblasts were treated with cardiomyocyte-pacing medium subjected to 5-Hz pacing in the absence of cardiomyocytes (group 0P) or to non-paced cardiomyocyte-pacing medium (group 0) and analyzed by [^{3}H]thymidine incorporation (Fig. 4A), Western-blot (Fig. 4B) and real-time RT-PCR (Fig. 4C). No differences in proliferation, αSMA expression, collagen-1 or fibronectin-1 mRNA levels were seen between treatment groups. Expression of GAPDH (Table 2B) and 18S-rRNA was also unaltered (Table 3B).

3.5. Effect of excitation–contraction uncoupling in rapidly-paced cardiomyocytes

To dissociate the role of cardiomyocyte excitation and contraction in tachypaced cardiomyocyte effects, cardiomyocytes were paced in the presence and absence of the myosin II inhibitor, blebbistatin [24,25]. Blebbistatin has been shown to be an effective excitation–contraction uncoupler, suppressing cardiomyocyte mechanical function at a concentration of 5 μM without altering electrical properties [26]. Activity of the drug was confirmed by measuring intracellular Ca^{2+} transients and

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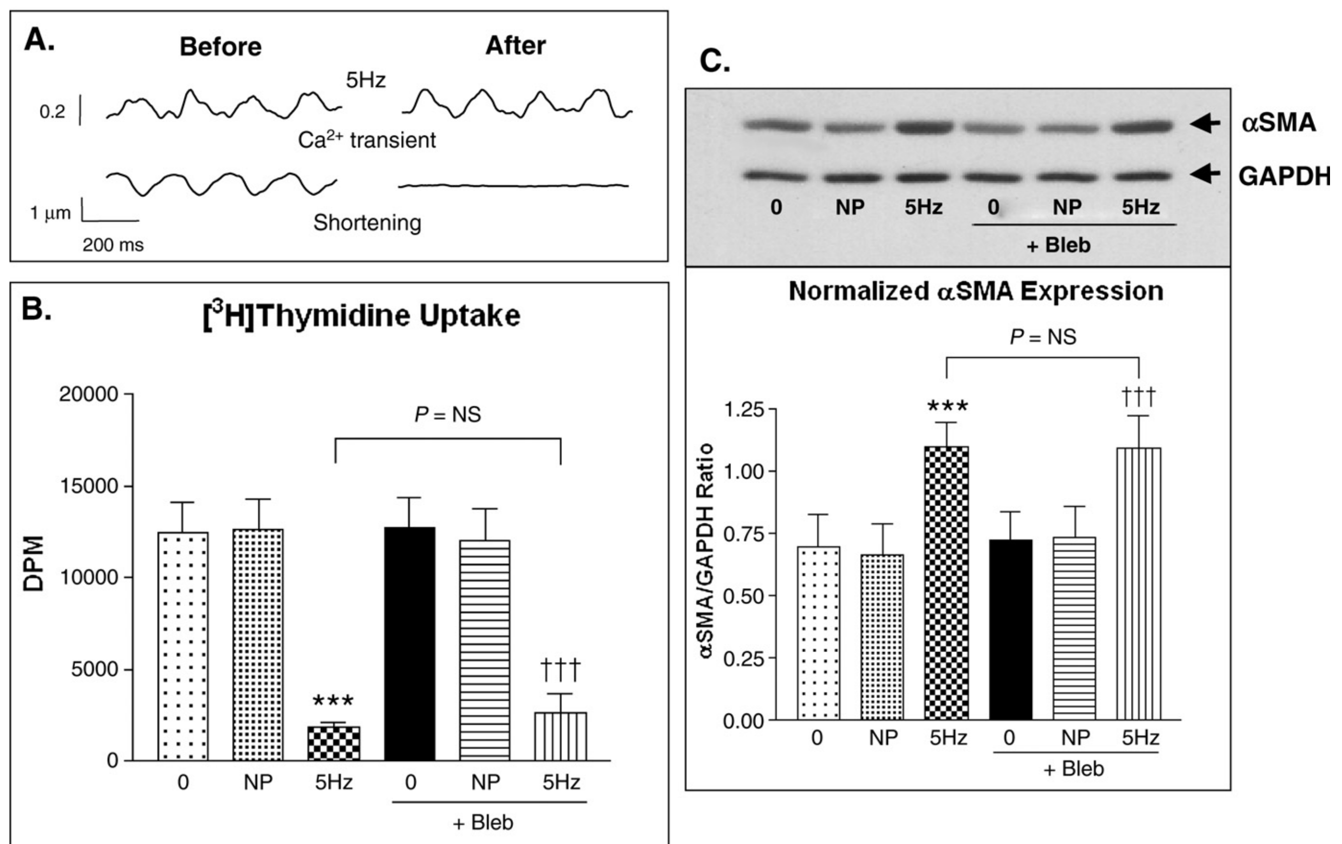

Fig. 5. A: Calcium transients and cell shortening in cultured cardiomyocytes, before and after treatment with blebbistatin (5 μM, $n=4$). Mean±SEM [^{3}H]Thymidine incorporation (B) and normalized αSMA expression (C, with representative αSMA/GAPDH immunoblot in upper panel) in fibroblasts treated with conditioned medium from cardiomyocytes paced in the presence or absence of blebbistatin; Bleb=blebbistatin, $n=6$/observation ***$p<0.001$ vs. basal and NP, $^{†††}p<0.001$ vs. basal+bleb and NP+bleb.

cell shortening in paced cardiomyocytes before and after addition of blebbistatin to the medium (Fig. 5A). The drug abolished contraction in treated cells, without affecting Ca^{2+} transients, confirming cell capture and separating excitation from contraction (n=4). Conditioned medium from myocytes tachypaced in the presence of blebbistatin significantly reduced [^{3}H]thymidine uptake (Fig. 5B) and increased αSMA expression (Fig. 5C, GAPDH values in Table 2C) in treated fibroblasts compared to fibroblasts treated with medium from blebbistatin-exposed non-paced cells and cell-free medium. Furthermore, the effects of blebbistatin-incubated tachypaced cardiomyocyte medium were indistinguishable from those of vehicle-incubated cell medium (Fig. 5B and C), confirming that tachypacing effects were unaffected by the dissociation of excitation and contraction in the rapidly-paced cardiomyocytes (n=6/observation).

3.6. Effects of angiotensin II type 1 (AT1) receptor inhibition on the responses to rapidly-paced cardiomyocyte-conditioned medium

Rapid pacing of cultured cardiomyocytes induces expression and secretion of the pro-fibrotic agent angiotensin II [27]. To elucidate the potential role of angiotensin II signaling, the AT1 receptor blocker valsartan was added to the rapidly-paced cardiomyocyte-conditioned medium before fibroblast exposure, at a concentration (10 μM) demonstrated to completely inhibit the action of up to 100 nM angiotensin II in several cell types [28–30]. The anti-proliferative effect of the conditioned medium was unaffected by valsartan (Fig. 6A). The conditioned medium effect to increase αSMA expression (Fig. 6B) was attenuated compared to vehicle-treated controls (1.00±0.10 vs. 1.52±0.14, n=6, $P<0.001$), without altering GAPDH (Table 2D). However, even in the presence of valsartan medium from tachypaced cardiomyocytes continued to enhance αSMA expression relative to non-paced cardiomyocyte medium (0.49±0.07, n=6) and cell-free medium (0.50±0.09, n=6, $P<0.001$ vs. tachypaced myocyte medium for each).

3.7. In vivo ECM-gene expression in response to atrial-tachypacing

One week of experimental ATP produces a clinically-relevant model of AF-related remodeling [9,16,31]. To determine whether similar changes in ECM genes occur *in vivo*, collagen-1 and fibronectin-1 mRNA expression was compared in ATP dogs (n=10) and unpaced controls (n=8). ATP significantly increased both collagen-1 (2.59±0.48 vs. 0.68±0.30, $P<0.01$) and fibronectin-1 (4.87±0.64 vs. 1.13±0.39, $P<0.001$) mRNA expression (Fig. 7A and B) without altering 18S-rRNA expression (Table 3C). αSMA protein expression (Fig. 7C) was also increased by ATP (1.35±0.07 vs. 0.98±0.13, n=7/group, $P<0.05$), without change in GAPDH expression (Table 2E). The *in vivo* increases in collagen-1 (~3.8-fold) and fibronectin-1 (~4.3-fold) support the *in vitro* observation that rapid rates of cardiomyocyte activation

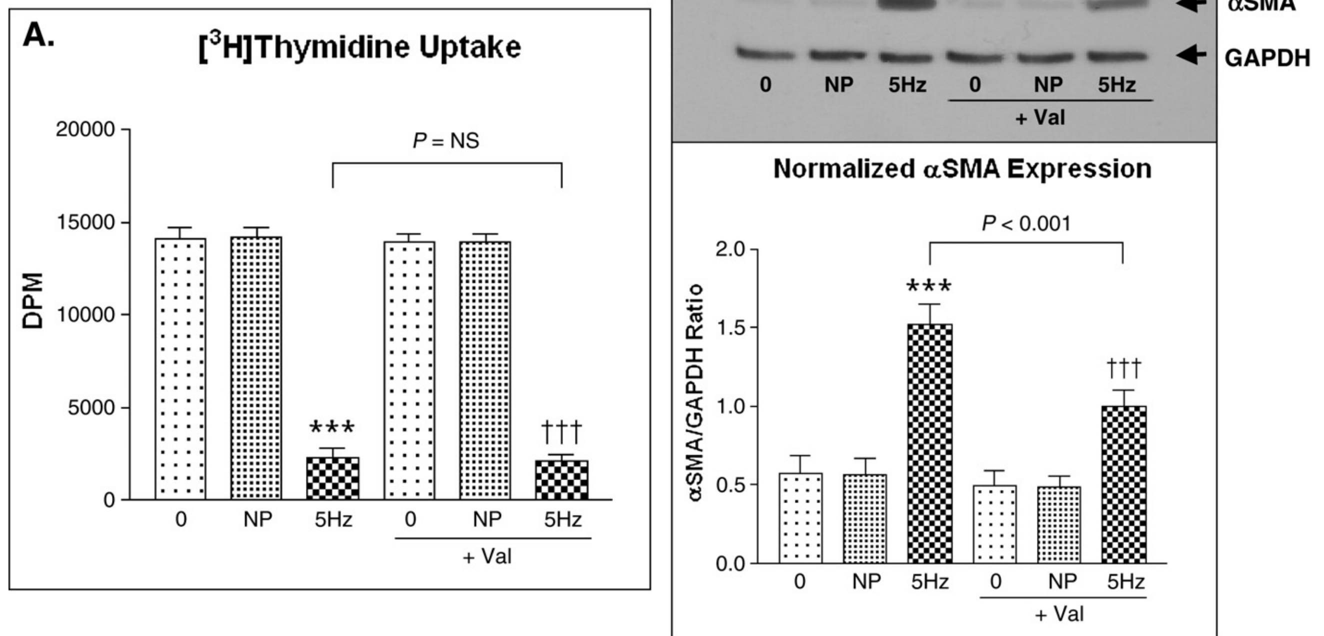

Fig. 6. Mean±SEM [^{3}H]Thymidine incorporation (B) and normalized αSMA expression (C, with representative αSMA/GAPDH immunoblot in upper panel) in fibroblasts treated with conditioned medium in the presence or absence of valsartan; Val=valsartan, n=6/observation $^{***}p<0.001$ vs. basal and NP, $^{†††}p<0.001$ vs. basal+Val and NP+Val.

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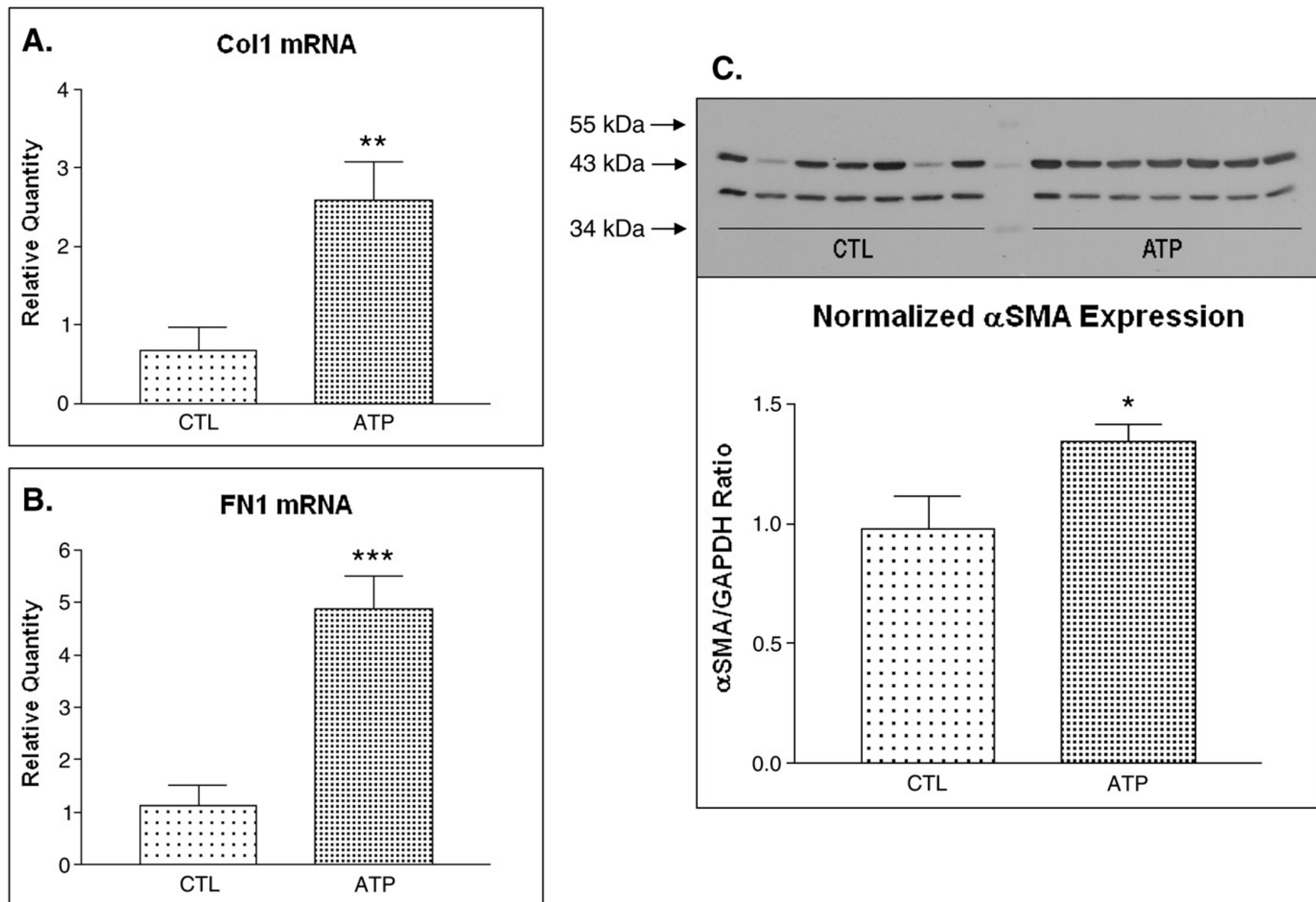

Fig. 7. Mean±SEM relative quantity of Col1 (A) and FN1 (B) mRNA in 1-week atrial-tachypaced dogs (ATP, $n=10$) and unpaced controls (CTL, $n=8$). C: Normalized αSMA expression (lower panel, $n=7$/group) and representative αSMA/GAPDH immunoblot (upper panel, with molecular weight markers in center lane separating groups; corresponding MWs indicated with arrows at left)- αSMA is upper band, GAPDH lower band; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

modulate cardiac fibroblast behavior, increasing ECM-protein secretory function.

4. Discussion

4.1. Main findings

We have demonstrated that rapid electrical stimulation of cardiomyocytes produces factors that induce a differentiated myofibroblast phenotype, characterized by reduced proliferation and increased secretory activity. This finding suggests that rapid atrial activation may influence structural remodeling by altering fibroblast function, and may contribute to the upregulation of ECM genes in response to atrial tachycardia.

4.2. Potential mechanisms

Our findings are consistent with previous studies showing that cardiomyocyte–fibroblast interactions can directly promote fibroblast secretory function [7,8], and relate to the central paracrine role of myofibroblasts [32]. Interestingly, the importance of the interaction between cardiomyocytes and fibroblasts in fibrillating atria has been suggested recently, based on observations on atrial myocytes from AF patients [33]. Tachypacing causes cardiomyocyte Ca^{2+}-loading, which can threaten cellular viability [6,34,35]. Conditions of impaired cell-viability are associated with fibrosis in various animal models [36,37]. The results of experiments with the excitation–contraction uncoupler blebbistatin reinforce the notion of an electrically-mediated process, and indicate that contraction *per se* is not needed to stimulate production of fibroblast-regulating substances from rapidly-firing cardiomyocytes. Various pro-fibrotic stimuli are known to induce a myofibroblast phenotype with enhanced secretory activity and reduced proliferation [23,38], resembling the activated cell type involved in ECM remodeling and fibrosis [39,40]. This phenotype is difficult to detect *in vivo*, due to fibroblast proliferation related at least in part to active recruitment and chemotaxis [41], but has been observed in cardiac fibroblasts of $TGF\beta_1$-overexpressing transgenic mice [42]. Similarly, intravenous $TGF\beta_1$ produces myocardial fibrosis with reduced fibroblast DNA synthesis [43].

Locally produced angiotensin II has been well established to exacerbate structural remodeling, and angiotensin II converting enzyme (ACE) inhibition attenuates the appearance of atrial fibrosis [44]. Cardiomyocytes have been

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shown to possess the machinery to synthesize angiotensin II [45], which is stimulated in cardiomyocytes when paced *in vivo* [46], and stretched *in vitro* [47]. Rapid pacing of cultured cardiomyocytes has been shown to result in significantly increased levels of expression and secretion of angiotensin II [27]. AT1-receptor blockade experiments with valsartan indicated that local production of angiotensin II plays a relatively minor role in the paced cardiomyocyte-induced myofibroblast differentiation, with valsartan only partially attenuating changes in secretory indices and showing no effect on proliferation. Other mediators including TGFβ_1, endothelin-1, TNFα and natriuretic peptides, acting alone or in combination, are potential candidates to contribute to the effects of tachypaced cardiomyocytes on fibroblast function. Further studies of the potential factor(s) involved would be of great interest but are beyond the scope of the present study.

4.3. Atrial structural remodeling as a potential consequence of AF

Electrical and structural remodeling are important contributors to the AF substrate [1,31,48]. Atrial fibrosis is common in clinical AF and in AF-promoting animal models of heart failure [1,48]. AF causes atrial cardiomyocyte cell-ultrastructural remodeling [48–50]; however, it is presently unknown whether atrial tachycardia/AF can promote fibrosis in the absence of ventricular dysfunction. Frustaci et al. have documented inflammatory infiltrates, myocyte hypertrophy, myocyte degeneration and fibrosis of unknown etiology in patients with lone AF [51]. Boldt et al. have shown increased ECM deposition in lone-AF patients, as well as in mitral valve disease patients with AF versus those without [52]. Furthermore, among patients with end-stage heart failure, collagen volume fraction is positively correlated with permanence of AF [53]. These findings are consistent with the notion that AF may promote fibrosis, although it is also possible that the association is due to a greater likelihood of AF with underlying fibrosis-inducing conditions.

Ausma et al. have extensively studied structural remodeling in experimental lone AF [48–50,54,55], and have described cellular changes resembling those seen in hibernating ventricular myocardium, characterized by cardiomyocyte volume increase, myolysis, glycogen accumulation, mitochondrial changes and chromatin redistribution. They have shown increased αSMA expression and ECM accumulation after 4 months of ATP [54], and clear fibrosis after 18 months [55]. Similarly, Chiu et al. have reported greater ECM content throughout the atria of chronic ATP dogs [56], and elevated collagen-1 [57] and fibronectin-1 [58] protein deposition has been documented in a porcine model of ATP. We have previously published a genomic analysis of canine ATP-remodeling revealing qualitatively-similar but statistically non-significant ECM-gene expression changes at 24 h and 1 week [15, on-line supplement].

4.4. Novelty and potential significance

To our knowledge, this is the first reported study of the effects of rapid cardiomyocyte activation on fibroblast function. The changes we observed would be expected to have offsetting effects on atrial pro-fibrotic tendencies: reduced fibroblast proliferation would tend to reduce fibrosis, whereas increased ECM-protein synthesis tends to enhance fibrosis. These offsetting changes may explain why overt atrial fibrosis is not observed after 1-week ATP with a controlled ventricular rate [1], despite the increased ECM-protein synthetic function we show here for 1-week ATP tissue. On the other hand, under conditions that promote the migration of fibroblast progenitors into rapidly activating atria (e.g. CHF, atrial stretch, diastolic dysfunction, etc), the decreased proliferation of atrial fibroblasts caused by ATP may be less relevant than the associated increase in ECM-protein secretion, and enhanced fibrosis could result. Since AF can cause impaired myocardial function, as well as atrial dilation, prolonged AF in itself could lead to atrial fibrosis. Our findings underscore the potential importance of normalizing cardiac rhythm to prevent irreversible AF-associated arrhythmogenic structural remodeling. Conversely, if long-standing sustained AF does not by itself ultimately produce fibrosis despite the observed ECM-gene upregulation, this would imply that the myocardium can adapt to counteract this signal (possibly by inhibiting fibroblast proliferation). An understanding of the underlying mechanisms may provide clues to novel therapeutic targets for the prevention of maladaptive cardiac fibrosis. Our results thus provide new insights into cardiomyocyte-fibroblast interactions in the fibrillating atrium that may have substantial clinical relevance and importance.

4.5. Limitations of the study

The *in vitro* experiments analyzed fibroblast response after only 24 h of treatment, from myocytes rapidly paced at one frequency, and may not apply to other frequencies or tachypacing durations. The culture conditions do not entirely replicate the *in vivo* environment, in which the development of fibrosis is regulated by the complex interplay among multiple resident myocyte and non-myocyte cell types, each subject to increased rate, mechanical and electrical influences beyond those of the *in vitro* system. Native canine cardiomyocytes were not used in the present study, as preliminary experiments revealed that variable cell survival, adhesion and capture rates did not allow reproducible conditioning of the medium. Instead, we elected to use HL-1 cardiomyocytes, a well characterized atrial-derived murine cell-line, shown to recapitulate the primary characteristics of ATP-induced remodeling *in vivo* [6]. We have not isolated an individual factor through which rapidly-activated cardiomyocytes influence fibroblast function, and given the possibility of multiple secreted agents acting in concert, this will likely be an important and challenging objective for future studies. Finally, factors other than rapid atrial rates, such as atrial-ventricular dyssynchrony, may contribute to AF effects on the ECM, but are beyond the scope of the present study.

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Acknowledgments

Valsartan was kindly provided by Novartis, Montreal, Canada. BB received a Canadian Institutes of Health Research (CIHR) MD/PhD studentship. AC is a Chercheur-boursier senior of the «Fonds de la recherche en santé du Québec». The authors thank Denis Chartier, Sabrina Le Bouter, Nathalie L'Heureux, Chantal St-Cyr, and Louis Villeneuve for technical assistance, and France Thériault for secretarial support.

References

[1] Li D, Fareh S, Leung TK, Nattel S. Promotion of atrial fibrillation by heart failure in dogs: atrial remodeling of a different sort. Circulation 1999;100:87–95.
[2] Shinagawa K, Shi YF, Tardif JC, Leung TK, Nattel S. Dynamic nature of atrial fibrillation substrate during development and reversal of heart failure in dogs. Circulation 2002;105:2672–8.
[3] Kannel WB, Abbott RD, Savage DD, McNamara PM. Epidemiologic features of chronic atrial fibrillation: the Framingham study. N Engl J Med 1982;306:1018–22.
[4] Andersen JS, Egeblad H, Abildgaard U, Aldershvile J, Godtfredsen J. Atrial fibrillation and left atrial enlargement: cause or effect? J Intern Med 1991;229:253–6.
[5] Thamilarasan M, Klein AL. Factors relating to left atrial enlargement in atrial fibrillation: "chicken or the egg" hypothesis. Am Heart J 1999;137: 381–3.
[6] Yang Z, Shen W, Rottman JN, Wikswo JP, Murray KT. Rapid stimulation causes electrical remodeling in cultured atrial myocytes. J Mol Cell Cardiol 2005;38:299–308.
[7] Pathak M, Sarkar S, Vellaichamy E, Sen S. Role of myocytes in myocardial collagen production. Hypertension 2001;37:833–40.
[8] Sarkar S, Vellaichamy E, Young D, Sen S. Influence of cytokines and growth factors in ANG II-mediated collagen upregulation by fibroblasts in rats: role of myocytes. Am J Physiol Heart Circ Physiol 2004;287:H107–17.
[9] Sun H, Gaspo R, Leblanc N, Nattel S. Cellular mechanisms of atrial contractile dysfunction caused by sustained atrial tachycardia. Circulation 1998;98:719–27.
[10] Claycomb WC, Lanson Jr NA, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, et al. HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci U S A 1998;95:2979–84.
[11] Brundel BJ, Shiroshita-Takeshita A, Qi X, Yeh YH, Chartier D, van Gelder IC, et al. Induction of heat shock response protects the heart against atrial fibrillation. Circ Res 2006;99:1394–402.
[12] Brundel BJ, Henning RH, Ke L, van Gelder IC, Crijns HJ, Kampinga HH. Heat shock protein upregulation protects against pacing-induced myolysis in HL-1 atrial myocytes and in human atrial fibrillation. J Mol Cell Cardiol 2006;41:555–62.
[13] Shiroshita-Takeshita A, Brundel BJ, Burstein B, Leung TK, Mitamura H, Ogawa S, et al. Effects of simvastatin on the development of the atrial fibrillation substrate in dogs with congestive heart failure. Cardiovasc Res 2007;74:75–84.
[14] Coutu P, Chartier D, Nattel S. Comparison of Ca^{2+}-handling properties of canine pulmonary vein and left atrial cardiomyocytes. Am J Physiol Heart Circ Physiol 2006;291:H2290–300.
[15] Cardin S, Libby E, Pelletier P, Le Bouter S, Shiroshita-Takeshita A, Le Meur N, et al. Contrasting gene expression profiles in two canine models of atrial fibrillation. Circ Res 2007;100:425–33.
[16] Yue L, Melnyk P, Gaspo R, Wang Z, Nattel S. Molecular mechanisms underlying ionic remodeling in a dog model of atrial fibrillation. Circ Res 1999;84:776–84.
[17] Drapeau J, El Helou V, Clement R, Bel-Hadj S, Gosselin H, Trudeau LE, et al. Nestin-expressing neural stem cells identified in the scar following myocardial infarction. J Cell Physiol 2005;204:51–62.
[18] Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002;3:349–63.
[19] Bashey RI, Donnelly M, Insinga F, Jimenez SA. Growth properties and biochemical characterization of collagens synthesized by adult rat heart fibroblasts in culture. J Mol Cell Cardiol 1992;24: 691–700.
[20] Bing OH, Ngo HQ, Humphries DE, Robinson KG, Lucey EC, Carver W, et al. Localization of alpha1(I) collagen mRNA in myocardium from the spontaneously hypertensive rat during the transition from compensated hypertrophy to failure. J Mol Cell Cardiol 1997;29: 2335–44.
[21] Pessanh MG, Mandarim-de-Lacerda CA. Myofibroblast accumulation in healing rat myocardium due to long-term low-dosage nitric oxide synthesis inhibition. Exp Toxicol Pathol 2000;52:192–4.
[22] Poobalarahi F, Baicu CF, Bradshaw AD. Cardiac myofibroblasts differentiated in 3D culture exhibit distinct changes in collagen I production, processing, and matrix deposition. Am J Physiol Heart Circ Physiol 2006;291:H2924–32.
[23] Hafizi S, Wharton J, Chester AH, Yacoub MH. Profibrotic effects of endothelin-1 via the ETA receptor in cultured human cardiac fibroblasts. Cell Physiol Biochem 2004;14:285–92.
[24] Straight AF, Cheung A, Limouze J, Chen I, Westwood NJ, Sellers JR, et al. Dissecting temporal and spatial control of cytokinesis with a myosin II inhibitor. Science 2003;299:1743–7.
[25] Allingham JS, Smith R, Rayment I. The structural basis of blebbistatin inhibition and specificity for myosin II. Nat Struct Mol Biol 2005;12: 378–9.
[26] Fedorov VV, Lozinsky IT, Sosunov EA, Anyukhovsky EP, Rosen MR, Balke WC, et al. Application of blebbistatin as an excitation–contraction uncoupler for electrophysiologic study of rat and rabbit hearts. Heart Rhythm 2007;4:619–26.
[27] Inoue N, Ohkusa T, Nao T, Lee JK, Matsumoto T, Hisamatsu Y, et al. Rapid electrical stimulation of contraction modulates gap junction protein in neonatal rat cultured cardiomyocytes: involvement of mitogen-activated protein kinases and effects of angiotensin II-receptor antagonist. J Am Coll Cardiol 2004;44:914–22.
[28] Nagata D, Takeda R, Sata M, Satonaka H, Suzuki E, Nagano T, et al. AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation. Circulation 2004;110: 444–51.
[29] Li JM, Cui TX, Shiuchi T, Liu HW, Min LJ, Okumura M, et al. Nicotine enhances angiotensin II-induced mitogenic response in vascular smooth muscle cells and fibroblasts. Arterioscler Thromb Vasc Biol 2004;24:80–4.
[30] Liu HW, Cheng B, Yu WL, Sun RX, Zeng D, Wang J, et al. Angiotensin II regulates phosphoinositide 3 kinase/Akt cascade via a negative crosstalk between AT1 and AT2 receptors in skin fibroblasts of human hypertrophic scars. Life Sci 2006;79:475–83.
[31] Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. Circulation 1995;92:1954–68.
[32] Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol 1999;277:C1–9.
[33] Rucker-Martin C, Pecker F, Godreau D, Hatem SN. Dedifferentiation of atrial myocytes during atrial fibrillation: role of fibroblast proliferation in vitro. Cardiovasc Res 2002;55:38–52.
[34] Kuramochi Y, Guo X, Sawyer DB, Lim CC. Rapid electrical stimulation induces early activation of kinase signal transduction pathways and apoptosis in adult rat ventricular myocytes. Exp Physiol 2006;91:773–80.
[35] Sun H, Chartier D, Leblanc N, Nattel S. Intracellular calcium changes and tachycardia-induced contractile dysfunction in canine atrial myocytes. Cardiovasc Res 2001;49:751–61.
[36] Nakajima H, Nakajima HO, Dembowsky K, Pasumarthi KB, Field LJ. Cardiomyocyte cell cycle activation ameliorates fibrosis in the atrium. Circ Res 2006;98:141–8.

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[37] Kajstura J, Zhang X, Liu Y, Szoke E, Cheng W, Olivetti G, et al. The cellular basis of pacing-induced dilated cardiomyopathy. Myocyte cell loss and myocyte cellular reactive hypertrophy. Circulation 1995;92: 2306–17.
[38] Agocha A, Sigel AV, Eghbali-Webb M. Characterization of adult human heart fibroblasts in culture: a comparative study of growth, proliferation and collagen production in human and rabbit cardiac fibroblasts and their response to transforming growth factor-beta1. Cell Tissue Res 1997;288:87–93.
[39] Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, et al. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. Am J Respir Cell Mol Biol 2001;24:591–8.
[40] Hetzel M, Bachem M, Anders D, Trischler G, Faehling M. Different effects of growth factors on proliferation and matrix production of normal and fibrotic human lung fibroblasts. Lung 2005;183:225–37.
[41] Mollmann H, Nef HM, Kostin S, von Kalle C, Pilz I, Weber M, et al. Bone marrow-derived cells contribute to infarct remodelling. Cardiovasc Res 2006;71:661–71.
[42] Nakajima H, Nakajima HO, Salcher O, Dittie AS, Dembowsky K, Jing S, et al. Atrial but not ventricular fibrosis in mice expressing a mutant transforming growth factor-beta(1) transgene in the heart. Circ Res 2000;86:571–9.
[43] Sigel A, Douglas JA, Eghbali-Webb M. Regulation of mRNA transcripts and DNA synthesis in the rat heart following intravenous injection of transforming growth factor beta 1. Mol Cell Biochem 1994;141:145–51.
[44] Cardin S, Li D, Thorin-Trescases N, Leung TK, Thorin E, Nattel S. Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and -independent pathways. Cardiovasc Res 2003;60:315–25.
[45] Zhang X, Dostal DE, Reiss K, Cheng W, Kajstura J, Li P, et al. Identification and activation of autocrine renin–angiotensin system in adult ventricular myocytes. Am J Physiol 1995;269:H1791–802.
[46] Barlucchi L, Leri A, Dostal DE, Fiordaliso F, Tada H, Hintze TH, et al. Canine ventricular myocytes possess a renin–angiotensin system that is upregulated with heart failure. Circ Res 2001;88:298–304.
[47] Malhotra R, Sadoshima J, Brosius III FC, Izumo S. Mechanical stretch and angiotensin II differentially upregulate the renin–angiotensin system in cardiac myocytes In vitro. Circ Res 1999;85:137–46.
[48] Allessie M, Ausma J, Schotten U. Electrical, contractile and structural remodeling during atrial fibrillation. Cardiovasc Res 2002;54:230–46.
[49] Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, Borgers M. Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. Circulation 1997;96:3157–63.
[50] Ausma J, Litjens N, Lenders MH, Duimel H, Mast F, Wouters L, et al. Time course of atrial fibrillation-induced cellular structural remodeling in atria of the goat. J Mol Cell Cardiol 2001;33:2083–94.
[51] Frustaci A, Chimenti C, Bellocci F, Morgante E, Russo MA, Maseri A. Histological substrate of atrial biopsies in patients with lone atrial fibrillation. Circulation 1997;96:1180–4.
[52] Boldt A, Wetzel U, Lauschke J, Weigl J, Gummert J, Hindricks G, et al. Fibrosis in left atrial tissue of patients with atrial fibrillation with and without underlying mitral valve disease. Heart 2004;90:400–5.
[53] Xu J, Cui G, Esmailian F, Plunkett M, Marelli D, Ardehali A, et al. Atrial extracellular matrix remodeling and the maintenance of atrial fibrillation. Circulation 2004;109:363–8.
[54] Ausma J, van der Velden HM, Lenders MH, van Ankeren EP, Jongsma HJ, Ramaekers FC, et al. Reverse structural and gap-junctional remodeling after prolonged atrial fibrillation in the goat. Circulation 2003;107: 2051–8.
[55] Thijssen VL, Ausma J, Liu GS, Allessie MA, van Eys GJ, Borgers M. Structural changes of atrial myocardium during chronic atrial fibrillation. Cardiovasc Pathol 2000;9:17–28.
[56] Chiu YT, Wu TJ, Wei HJ, Cheng CC, Lin NN, Chen YT, et al. Increased extracellular collagen matrix in myocardial sleeves of pulmonary veins: an additional mechanism facilitating repetitive rapid activities in chronic pacing-induced sustained atrial fibrillation. J Cardiovasc Electrophysiol 2005;16:753–9.
[57] Pan CH, Lin JL, Lai LP, Chen CL, Stephen Huang SK, Lin CS. Downregulation of angiotensin converting enzyme II is associated with pacing-induced sustained atrial fibrillation. FEBS Lett 2007;581: 526–34.
[58] Lin CS, Lai LP, Lin JL, Sun YL, Hsu CW, Chen CL, et al. Increased expression of extracellular matrix proteins in rapid atrial pacing-induced atrial fibrillation. Heart Rhythm 2007;4:938–49.

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