Hypercoagulability causes atrial fibrosis and promotes atrial fibrillation

European Heart Journal (2017) **38**, 38–50
doi:10.1093/eurheartj/ehw119

BASIC SCIENCE

Hypercoagulability causes atrial fibrosis and promotes atrial fibrillation

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Received 13 May 2015; revised 21 December 2015; accepted 2 March 2016; online publish-ahead-of-print 12 April 2016

See page 51 for the editorial comment on this article (doi:10.1093/eurheartj/ehw108)

Aims Atrial fibrillation (AF) produces a hypercoagulable state. Stimulation of protease-activated receptors by coagulation factors provokes pro-fibrotic, pro-hypertrophic, and pro-inflammatory responses in a variety of tissues. We studied the effects of thrombin on atrial fibroblasts and tested the hypothesis that hypercoagulability contributes to the development of a substrate for AF.

Methods and results In isolated rat atrial fibroblasts, thrombin enhanced the phosphorylation of the pro-fibrotic signalling molecules Akt and Erk and increased the expression of transforming growth factor β1 (2.7-fold) and the pro-inflammatory factor monocyte chemoattractant protein-1 (6.1-fold). Thrombin also increased the incorporation of ^{3}H-proline, suggesting enhanced collagen synthesis by fibroblasts (2.5-fold). All effects could be attenuated by the thrombin inhibitor dabigatran. In transgenic mice with a pro-coagulant phenotype ($TM^{pro/pro}$), the inducibility of AF episodes lasting >1 s was higher (7 out of 12 vs. 1 out of 10 in wild type) and duration of AF episodes was longer compared with wild type mice (maximum episode duration 42.8 ± 68.4 vs. 0.23 ± 0.39 s). In six goats with persistent AF treated with nadroparin, targeting Factor Xa-mediated thrombin generation, the complexity of the AF substrate was less pronounced than in control animals (LA maximal activation time differences 23.3 ± 3.1 ms in control vs. 15.7 ± 2.1 ms in nadroparin, $P < 0.05$). In the treated animals, AF-induced α-smooth muscle actin expression was lower and endomysial fibrosis was less pronounced.

Conclusion The hypercoagulable state during AF causes pro-fibrotic and pro-inflammatory responses in adult atrial fibroblasts. Hypercoagulability promotes the development of a substrate for AF in transgenic mice and in goats with persistent AF. In AF goats, nadroparin attenuates atrial fibrosis and the complexity of the AF substrate. Inhibition of coagulation may not only prevent strokes but also inhibit the development of a substrate for AF.

Keywords Atrial fibrillation • Hypercoagulability • Stroke • Protease-activated receptors • Direct thrombin inhibitors • Atrial fibrosis

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Translational perspective

This article describes experimental evidence for the fact that hypercoagulability—as caused by atrial fibrillation—induces pro-fibrotic and pro-inflammatory responses in adult atrial fibroblasts, that transgenic mice with hypercoagulability are more susceptible to atrial fibrillation than wild-type mice, and that the inhibition of hypercoagulability can partially prevent atrial remodelling in a well-characterized animal model of AF. These data show that the traditional causality of AF causing hypercoagulability may also hold in the opposite direction, i.e. that hypercoagulability has the potential to cause or promote AF. If confirmed in human studies, this new paradigm may have consequences for anticoagulant therapy in patients with or at risk for AF.

Introduction

While the observation that atrial fibrillation (AF) causes activation of blood coagulation has been made decades ago, far less is known about the effect of activated blood coagulation on atrial remodelling and on the progression of AF. The discovery of protease-activated receptors (PARs) in the early 1990s and the identification of multiple coagulation proteases (i.e. clotting factors) as their main natural ligands triggered intense research on non-haemostatic functions of the coagulation cascade.[1–3] It was demonstrated that activation of PARs by coagulation factors can cause vascular alterations, pro-fibrotic effects, and inflammation.[1–3]

Protease-activated receptors belong to the transmembrane G protein-coupled receptors superfamily and are expressed in many different cell types throughout the body.[4,5] The four isoforms (PAR1 to PAR4) differ in their expression levels in various cell types and their downstream signalling.[6] PAR1 and PAR2 are the most important isoforms in the heart.[7] Thrombin, an important effector of the coagulation cascade, can activate PAR1, PAR3, and PAR4, while Factor Xa mainly activates PAR1 and PAR2.

PAR1 is expressed in myocytes, fibroblasts, endothelial cells, and smooth muscle cells.[4] Its expression is elevated in left ventricular tissue from patients with ischaemic heart disease or heart failure.[8,9] PAR1 activation leads to myocyte hypertrophy[6,7] and proliferation of fibroblasts.[10,11] Mice overexpressing PAR1 develop cardiac hypertrophy and dilated cardiomyopathy.[12] In contrast, PAR1 deficiency was protective in a mouse model of ischaemia–reperfusion.[12]

PAR2 is expressed by myocytes, smooth muscle cells, and endothelial cells, but its expression by fibroblasts is uncertain.[4] In myocytes, PAR2 activation leads to mitogen-activated protein kinase activation and cellular hypertrophy.[7] PAR2 deficiency leads to reduced infarct sizes in ischaemia–reperfusion models and reduces inflammatory responses.[8]

Outside the heart, an important role of PARs has been implicated in many fibro-proliferative diseases such as fibrosis of skin, lung, and liver.[13–15] It is so far unclear to what extent PAR activation by the coagulation system could contribute to fibrosis in the heart. As inflammatory changes, tissue fibrosis, and cellular hypertrophy significantly contribute to loss of electrical conductivity between myocytes and thus conduction disturbances in dilated and fibrillating atria,[16] we hypothesize that the hypercoagulable state during AF promotes structural remodelling in the atria and contributes to the development of a substrate for AF by PAR activation. We aimed to investigate whether activated coagulation factors induce fibroblast remodelling, whether hypercoagulability enhances the inducibility and stability of AF episodes in transgenic mice, and whether nadroparin, targeting Factor Xa and thrombin, inhibits the development of a substrate for AF in goats.

Materials and methods

Isolation and culture of adult rat atrial fibroblasts

Atria of adult rats were excised and placed in ice-cold Krebs Henseleit buffer. The tissue was minced into small pieces and digested with 0.15 mg/mL Liberase TM (Roche Diagnostics GmbH, Mannheim, Germany). Cells from the digestions were pooled, filtered, and centrifuged. The pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) with 10% foetal calf serum (FCS) and penicillin–streptomycin (50 U/mL and 50 mg/mL, respectively) (Invitrogen Corporation, Breda, The Netherlands) and seeded on uncoated dishes.[17,18] On the next day, non-adherent cells were removed by washing with phosphate buffered saline (PBS). Cells were cultured at 37°C under 5% CO_2 in DMEM with 10% FCS and 1% penicillin–streptomycin (50 U/mL and 50 mg/mL, respectively) (Invitrogen Corporation, Breda, The Netherlands). At confluence, cells were split using 0.05% trypsin-ethylenediaminetetraacetic acid (Life technologies, Bleiswijk, The Netherlands) and cultured again until confluence was reached. After this step, cells were trypsinized, counted, and seeded at a density of 15 000 cells/cm^2. On the next day, cells were serum starved for 24 h before experiments were carried out. Cells showed positive staining for vimentin (Santa Cruz Biotechnology, Heidelberg, Germany) and prolyl 4-hydroxylase subunit beta (P4HB, Novus Biologicals, Littleton, CO, USA), and part of the cells were positive for smooth muscle actin (Dako, Heverlee, Belgium), indicative of differentiation towards myofibroblasts. Less than 5% of the cells were negative for P4HB.

Cell stimulation with thrombin

Cells were exposed to 10^{-5} units/mL up to 0.5 units/mL thrombin from human plasma (Diagnostica Stago S.A.S., Asnières sur Seine, France) for 5 min up to 24 h. Dabigatran (Boehringer Ingelheim) at a final concentration of 350 ng/mL was added 30 min before the addition of thrombin. The dabigatran concentration was based on approximately two times the maximum plasma concentration in patients and the concentration required to reduce *in vitro* plasma thrombin generation by 50% (data not shown). To investigate whether the responses induced via thrombin were mediated by PAR1, we used PAR1 antagonists, 30–100 μM RWJ56110 (Tocris Bioscience, Bristol, UK) and 1 μM SCH79797 dihydrochloride (Tocris Bioscience, Bristol, UK). Furthermore, the PAR1 blocking antibody ATAP2 (Santa Cruz Biotechnology, Heidelberg, Germany) was used at 10 μg/mL. The PAR1 receptor agonist Trap-14 (Bachem, Bubendorf, Switzerland) was used as a positive control.

Western blots

Proteins from whole cell lysates were isolated using radioimmunoprecipitation assay buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Igepal ca-630 in Tris-buffered saline). After denaturation at 99°C for 5 min, proteins were separated by SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membranes (Bio-Rad, Veenendaal, The Netherlands) and blocked with 5% milk. Blots were incubated with antibodies against phospho-Erk (E-4) (Santa Cruz Biotechnology, Heidelberg, Germany), phospho-Akt (Ser473) (D9E), or phospho-p38 (Thr180/Tyr182) (both Cell Signaling Technology, Danvers, MA, USA). The membranes were stripped (stripping buffer containing 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris buffer) for 30 min at 55°C, washed, and incubated with the corresponding antibody detecting the total protein amount, i.e. total p44/42 MAPK (Erk1/2) (137F5) and Akt (C67E7) and p38 (all three Cell Signalling Technology, Danvers, MA, USA). After that, membranes were reprobed with GAPDH antibodies (Fitzgerald Industries International, Acton, MA, USA). Signals were detected using enhanced chemiluminescence (Perkin Elmer, Waltham, MA, USA) and quantified by densitometry. Protein levels were expressed relative to the respective control group of each experiment.

Real-time quantitative polymerase chain reaction

Total RNA isolated using the Nucleospin II kit (Macherey-Nagel, Düren, Germany) was converted to cDNA by the QuantiTect Reverse Transcription kit (Qiagen, Venlo, The Netherlands). Gene expression was measured with Absolute QPCR SYBR Green ROX Mix (Abgene, Epsom, UK) in the presence of 7.5 ng cDNA and 200 nM forward and reverse primers. Real-time quantitative PCR was performed on a Bio-Rad CFX384 cycler. The initial denaturation and activation of the DNA polymerase (95°C for 3 min) was followed by 35 cycles with denaturation for 15 s at 95°C and annealing and elongation for 30 s at 60°C, followed by a melt curve. Gene expression levels were corrected for ribosomal protein P0 (36b4) reference gene expression, and values were expressed relative to the respective control group of each experiment. Primers used are shown in *Table 1*.

Proline incorporation

Cells were grown in 12-well plate and serum starved for 24 h. L-[2,3,4,5-^{3}H]Proline (Perkin Elmer, Groningen, The Netherlands) was added to all wells at a concentration of 1 μCi/mL directly after adding thrombin. Cells were cultured for an additional 24 h. Thereafter, cells were washed twice with PBS, and proteins were solubilized with 0.5 M NaOH for 1 h at room temperature. Proteins were collected in scintillation tubes, and 4 mL of Ultima Gold XR scintillation liquid (Perkin Elmer, Groningen, The Netherlands) was added. The amount of radioactivity was determined by a LS6500 Beckman counter.

Table 1 Primers

Gene	Forward	Reverse
36b4	gttgcctcagtgcctcactc	gcagccgcaaatgcagatgg
IL6	aacagcgatgatgcactgtc	tccaggtagaaacggaactc
MCP-1	ccgactcattgggatcatctt	tgtctcagccagatgcagttaat
PAR1	ttcctggcagtggtgtaccc	cgtggcaggtggtgatgttg
PAR2	tgggaggtatcacccttctg	gcgtgtccaatctgccaatc
PAR3	gggttaggctgctgtttctg	tagttgtggtggctcctgtc
PAR4	atgctcgggttcagcatcag	cctcgtggattaggcttgtc
Smooth muscle actin	caccatcgggaatgaacg	tgcctgggtacatggtag
TGFβ1	aagaagtcacccgcgtgcta	tgtgtgatgtctttggttttgtca

IL6, interleukin-6; MCP-1, monocyte chemotactic protein-1; PAR, protease-activated receptor; TGFβ1, transforming growth factor β1.

Immunofluorescence and proliferation

Cells were fixated in 4% paraformaldehyde for 10 min on ice and washed with PBS. Proliferaton was detected using the CyQuant cell proliferation assay kit (Invitrogen–Molecular Probes, Eugene, OR, USA). To confirm fibroblast phenotype, a counter staining with P4HB was performed.

Inducibility and stability of atrial fibrillation in transgenic mice with enhanced thrombin activity

TM$^{pro/pro}$ mice carry a thrombomodulin (TM) gene mutation which causes diminished generation of activated protein C (APC),[19] resulting in a hypercoagulable phenotype.[20] Adult mice [wild type (WT), $n = 10$, weight 24.9 ± 4.5 g; and TM$^{pro/pro}$, $n = 12$, weight 24.2 ± 2.2 g] were anaesthetized by inhalation of 2% isoflurane through a nose cone. An octapolar catheter (CIBer, NuMed Inc., Hopkinton, NY, USA) was inserted in the oesophagus and connected to a stimulus isolation unit. At an output of two times threshold, pacing protocols were applied to determine the sinus node recovery time [interval from the last stimulus of a train of 40 stimuli with a basic cycle length (BCL) of 100 ms to the onset of the first subsequent P wave] and atrioventricular Wenckebach cycle length (AV Wenck, first cycle length where 1:1 conduction to the ventricles was lost when the BCL was decreased from 100 ms by steps of 2 ms). Atrial fibrillation inducibility was tested by applying 5 s trains of stimuli with a cycle length starting at 60 ms and decreased by 2 ms for each successive train. Atrial fibrillation was defined as a rapid irregular atrial rhythm with an irregular ventricular response lasting longer than 1 s. Atrial tissues were excised, fixated in buffered 4% formaldehyde, and embedded in paraffin. Tissue sections of 5 μm were deparaffinized, rehydrated, and stained with Sirius red for collagen deposition. Photographs were taken at ×100 magnification, and collagen staining was quantified using ImageJ analysis software.

Effect of nadroparin treatment on the development of an atrial fibrillation substrate in goats

In 15 female goats, an atrial endocardial pacemaker lead (Medtronic Capsurefix®) was implanted in the right atrial appendage and connected to an implantable neurostimulator (Medtronic Itrel®). Atrial fibrillation was maintained by repetitive 50 Hz burst pacing at three times threshold. Six goats were treated with subcutaneous injections of 150 IU/kg of the low molecular weight heparin nadroparin twice daily during the entire AF stimulation period. Nine untreated animals served as controls. After 4 weeks of AF, an open chest experiment was performed. Animals were anaesthetized with sufentanyl (6 μg/kg/h), midazolam (0.8 mg/kg/h), and pancuronium (0.3 mg/kg/h). After left-sided thoracotomy, mapping was performed as described recently.[21] Left and right AF electrograms were recorded for 30 s in each animal using a spoon-shaped epicardial mapping electrode (12.8 cm^2, 2.4 mm inter-electrode distance). A silver plate in the thoracic cavity served as indifferent electrode. Unipolar signals were recorded using a custom-made 256-channel mapping amplifier (bandwidth 0.1–408 Hz, sampling rate 1 kHz, resolution 16 bits) and stored for offline analysis. Thereafter, left and right atrial biopsies were taken for histological analysis.

Local activation times were identified as points of maximum negative d*V*/d*t* in each unipolar electrogram. Fibrillation waves were defined as areas surrounded by lines of block or fusion everywhere along their boundaries within the mapping array as described earlier.[22,23] To quantify AF complexity, we calculated the maximal activation time differences in each AF beat and the unipolar fractionation index as described previously.[24]

Thrombin generation in goat plasma

Thrombin generation in goat plasma was measured using the calibrated automated thrombogram (Thrombinoscope B.V., Maastricht, The Netherlands) using platelet-poor plasma using final concentrations of 1 pM tissue factor and 4 μM phospholipids.[25] To correct for inner-filter effects and substrate consumption, the results from each thrombin generation analysis were calibrated against the fluorescence curve obtained from the same plasma with a fixed amount of calibrator (Thrombin Calibrator, Thrombinoscope B.V., Maastricht, The Netherlands). Fluorescence was measured in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 nm filter set, and thrombin generation curves were calculated using Thrombinoscope software (Thrombinoscope B.V., Maastricht, The Netherlands). All coagulation measurements were performed in triplicate.

Histology

After the electrophysiological studies in goats, hearts were excised, and tissue samples were fixated in buffered 4% formaldehyde, and embedded in paraffin for histological evaluation. Tissue sections of 5 μm were de-paraffinized, rehydrated, and stained with haematoxylin and eosin. Photographs were taken at ×400 magnification. In each goat, the diameter of the atrial myocytes was measured in 30–35 myocytes, with the nucleus in plane of the section. Endomysial fibrosis was defined as connective tissue separating individual myocytes within bundles. The width of endomysial tissue septa was quantified as the smallest distance between myocytes within bundles. ImageJ 1.37a (National Institute of Health, USA) was used for analysis.

Staining for matrix metalloproteinase-1 (MMP-1) and for tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) was performed on frozen tissue sections with antibodies obtained from Bio-Connect Diagnostics (Huissen, The Netherlands) and for transforming growth factor β1 (TGFβ1; Santa Cruz Biotechnology, TX, USA) using a tyramide-FITC enhancement step as described.[26] Whole mount images were made with a ×20 objective using an automated slide scanner (3D-Histech, Budapest, Hungary). Whole mount images were generated with the program Pannoramic Viewer (3D-Histech, Boedapest, Hungary), and staining was quantified using ImageJ.

To quantify the number of myofibroblasts, α-smooth muscle actin (αSMA, Thermo Scientific, Rockford, IL, USA) staining was performed as described above. For visualization of the relatively small myofibroblasts, from each section at least three images were generated at higher magnification (×20; in total ×400) using Pannoramic Viewer. α-Smooth muscle actin-positive vascular smooth muscle cells of the larger vessels were manually excluded from the analysis by applying a maximal threshold in the analysis program ImageJ. Results were expressed as percentage of positive area per image.

All animal studies were conducted in accordance with national and institutional guidelines for the use of laboratory animals and were approved by the local ethics committees for animal experiments of the Universities in Groningen or Maastricht.

Statistics

Data are expressed as mean values ± standard deviation. Comparisons between two groups were done using a Student's *t*-test, and comparisons between multiple groups were done using one-way analysis of variance (ANOVA) with *post hoc* Dunnett correction. If parameters were investigated in both right and left atria, a two-way ANOVA was used. Two-sided *P*-values of <0.05 were considered statistically significant. Statistical analyses were performed using Graphpad Prism 6 for Mac OS X (Graphpad Software Inc.).

Results

Effects of thrombin on pro-fibrotic and pro-inflammatory pathways

Real-time PCR confirmed the presence of PAR1 mRNA in adult atrial fibroblasts. PAR2 and PAR3 mRNA expressions were at least 10-times lower (based on the number of PCR cycles required for the detection of the respective mRNAs), and PAR4 mRNA could not be detected in these cells.

Five minutes exposure of atrial fibroblasts to thrombin was sufficient to induce phosphorylation of the PAR signalling molecules Akt, Erk, and p38 (*Figure 1*). Incubation with dabigatran inhibited phosphorylation of these signalling molecules.

Thrombin exposure for 24 h increased mRNA levels of interleukin-6 (*Figure 2A*) and monocyte chemotactic protein-1 (MCP-1) (*Figure 2B*) in atrial fibroblasts. Thrombin also increased mRNA expression of αSMA (*Figure 3A*), which is indicative of differentiation towards active myofibroblasts. In addition, expression of TGFβ1 (*Figure 3B*) was increased. ^{3}H-proline incorporation (*Figure 3C*) was dose-dependently increased with thrombin, indicating increased collagen production. Again, dabigatran attenuated all these effects.

Pro-inflammatory and pro-fibrotic effects of thrombin are mediated by protease-activated receptor-1

Figure 4A shows the effect of PAR1 receptor antagonists on the thrombin-induced increase in ^{3}H-proline incorporation in atrial fibroblasts. The PAR1 receptor antagonists, RWJ56110 and SCH79797, and ATAP-2, a PAR1 blocking antibody, strongly attenuated the effect of thrombin on ^{3}H-proline incorporation. *Vice versa*, Trap-14, a PAR1 agonist, strongly increased the incorporation of ^{3}H-proline. This effect was inhibited by RWJ56110, SCH79797, and ATAP-2. Trap-14 enhanced gene expression of TGFβ1, αSMA, and MCP-1 to a similar extent as thrombin (*Figure 4B*). These data suggest that the response induced by thrombin is mediated by PAR1 activation. In addition, PAR1 mRNA expression was up-regulated when the cells were stimulated with thrombin (*Figure 4C*), indicating a positive feedback up-regulation of PAR1 induced by its stimulation with thrombin. The same response was found for the isoforms PAR2 and PAR3 (*Figure 4D* and *E*). However, the expression level of PAR2 and PAR3 was far lower than for PAR1. Dabigatran attenuated the response of the PARs on thrombin exposure.

Thrombin does not induce fibroblast proliferation

Thrombin did not induce proliferation, i.e. an increase in cell number of atrial fibroblasts. In contrast, FCS (positive control) did induce proliferation (*Figure 5*).

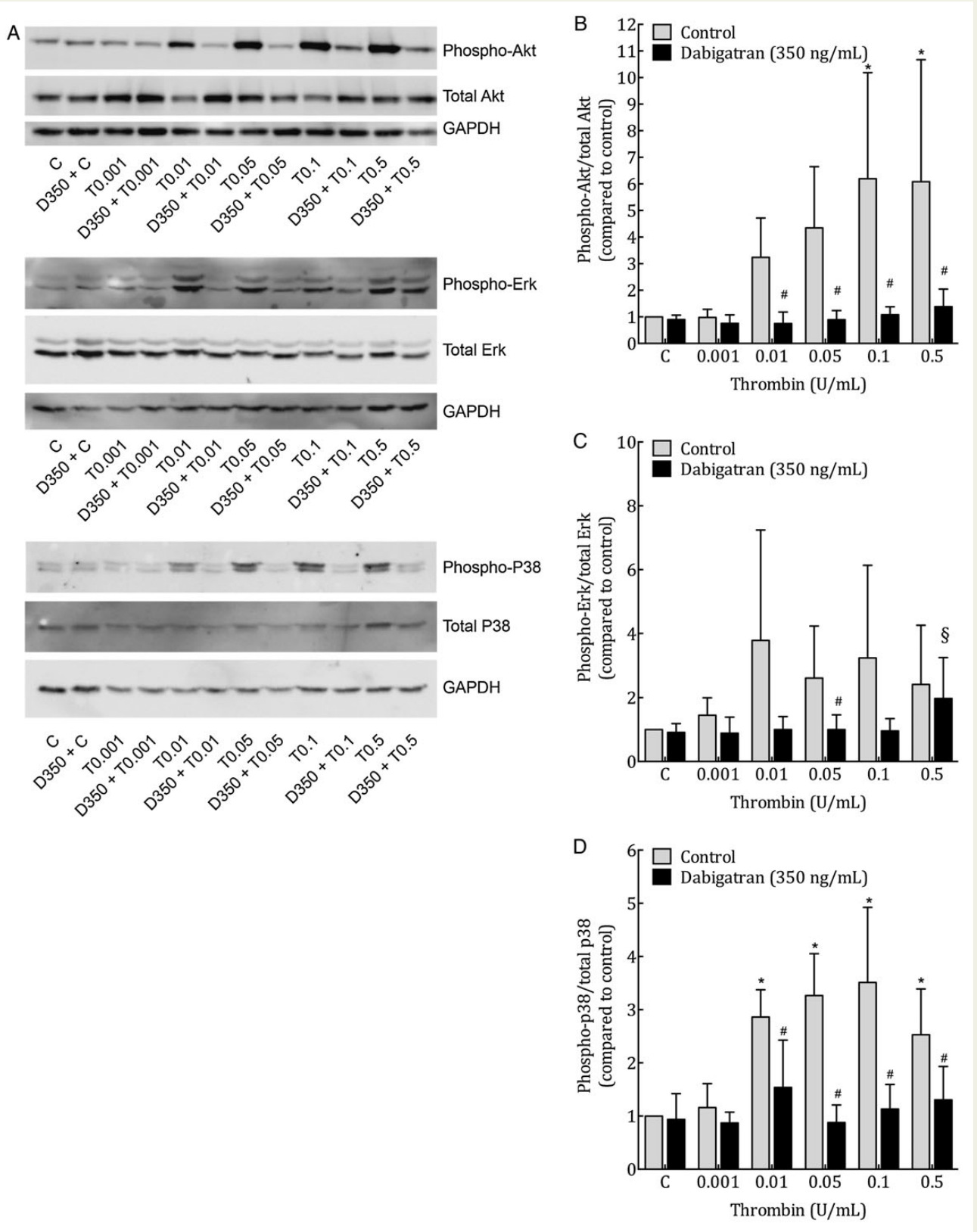

Figure 1 Thrombin induces phosphorylation of Erk ($n = 6–7$), Akt ($n = 7–8$), and p38 ($n = 5–6$). Dabigatran attenuated these effects. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with thrombin only; $^{\S}P < 0.05$ compared with dabigatran control.

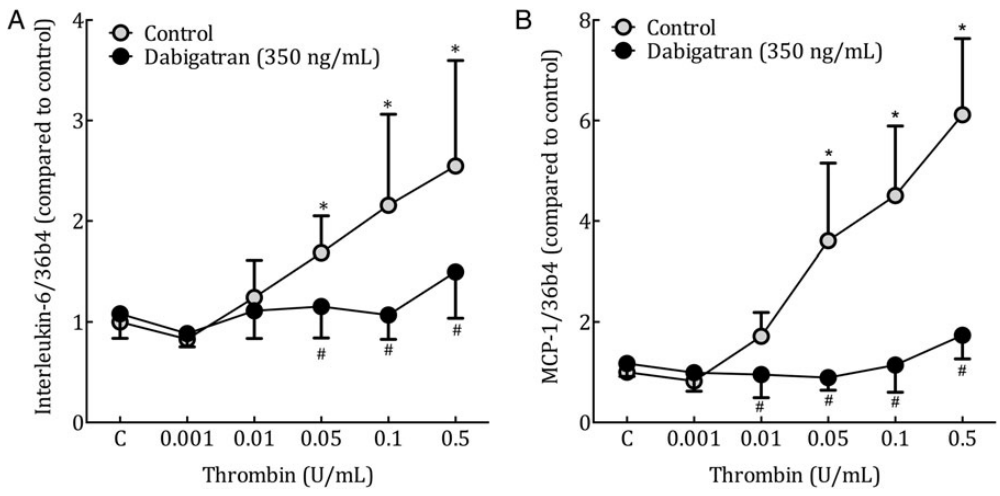

Figure 2 Thrombin dose-dependently increased expression of (*A*) interleukin-6 ($n = 8–13$) and (*B*) monocyte chemotactic protein-1 ($n = 8–12$). Dabigatran reduced these effects. IL6, interleukin-6; MCP-1, monocyte chemoattractant protein-1. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with thrombin only.

Inducibility and stability of atrial fibrillation in transgenic mice with enhanced thrombin activity

Mice with a hypercoagulable phenotype ($TM^{pro/pro}$) showed similar electrocardiogram intervals and indicators for SA and AV node function when compared with WT mice (*Figure 6A*, left and right panels, respectively). However, burst pacing evoked episodes of AF lasting longer than 1 s in a significantly larger proportion of $TM^{pro/pro}$ mice than in WT mice, and the duration of the longest AF episode observed in each mouse was also significantly longer in $TM^{pro/pro}$ mice (*Figure 6C*, example in *Figure 6B*). Enhanced inducibility of AF in the $TM^{pro/pro}$ mice was associated with increased collagen deposition in atria of the heart (*Figure 6D*). Compared with WT mice ($0.078\% \pm 0.025$), collagen area coverage was six-fold higher in $TM^{pro/pro}$ mice ($0.457\% \pm 0.114$, $P = 0.0182$).

Nadroparin inhibits the development of an atrial fibrillation substrate in goats

In goats with AF, the effect of treatment with nadroparin, targeting Factor Xa, and thrombin on the remodelling process was investigated. Before induction of AF, tissue factor-induced thrombin generation was 152 ± 23 nM*min in goat plasma. After 4 weeks of AF, tissue factor-induced thrombin generation had increased to 252 ± 44 nM*min confirming that 4 weeks of AF induces hypercoagulability in goats. Nadroparin treatment decreased thrombin generation by 75% demonstrating substantial inhibition of coagulation.

After 4 weeks of AF, a protective effect of nadroparin treatment was clearly detectable (*Figure 7*). The complexity of AF was lower in the nadroparin group. Maximal activation time differences measured with a mapping electrode (a commonly used measure for conduction heterogeneity and block)[27] were significantly shorter in the nadroparin group in both right and left atria (*Figure 7A*). Also, the unipolar fractionation index was lower in the nadroparin group (*Figure 7B*). There was no difference in AF cycle length between the two groups (*Table 2*). The cell–cell distances, an early marker for atrial endomysial fibrosis,[22] was ~50% lower in the nadroparin group in both right and left atria (*Figure 7C*). Similarly, the number of αSMA-positive myofibroblasts[28] was reduced in the treated animals (*Figure 7D*). The cell diameter of atrial myocytes was not affected by nadroparin treatment (RA: ctr 15.4 ± 3.6 μm, nadroparin 14.1 ± 2.6 μm; LA: ctr 16.9 ± 3.0 μm, nadroparin 15.1 ± 2.3 μm).

Anticoagulation through nadroparin not only affected AF-related fibrosis but influenced matrix degradation as well. Levels of MMP-1 and its inhibitor TIMP-1, as assessed by immunohistochemical analysis, were not significantly altered upon treatment with nadroparin. However, the difference between left and right atria (delta MMP-1) was reduced by a factor 9 through nadroparin administration ($P = 0.026$). The ratio of MMP-1 over TIMP-1 was not different between control and anticoagulated animals for the separate left and right atria. For the combined left and right atria, the MMP-1/TIMP-1 ratio was ~80% reduced in the atria of nadroparin-treated goats (*Figure 7E*). No differences were observed in TGFβ1 staining between treated and non-treated groups.

Discussion

The present study shows that thrombin induces pro-inflammatory and pro-fibrotic effects in adult atrial fibroblasts. These effects seem to be mediated by PAR1-receptor stimulation. Importantly, the effects of thrombin can be inhibited by the direct thrombin inhibitor dabigatran. In transgenic mice with enhanced thrombin generation due to a thrombomodulin mutation, inducibility and stability of AF episodes as well as atrial collagen deposition are enhanced. Finally, nadroparin treatment, targeting Factor Xa-mediated thrombin generation, is

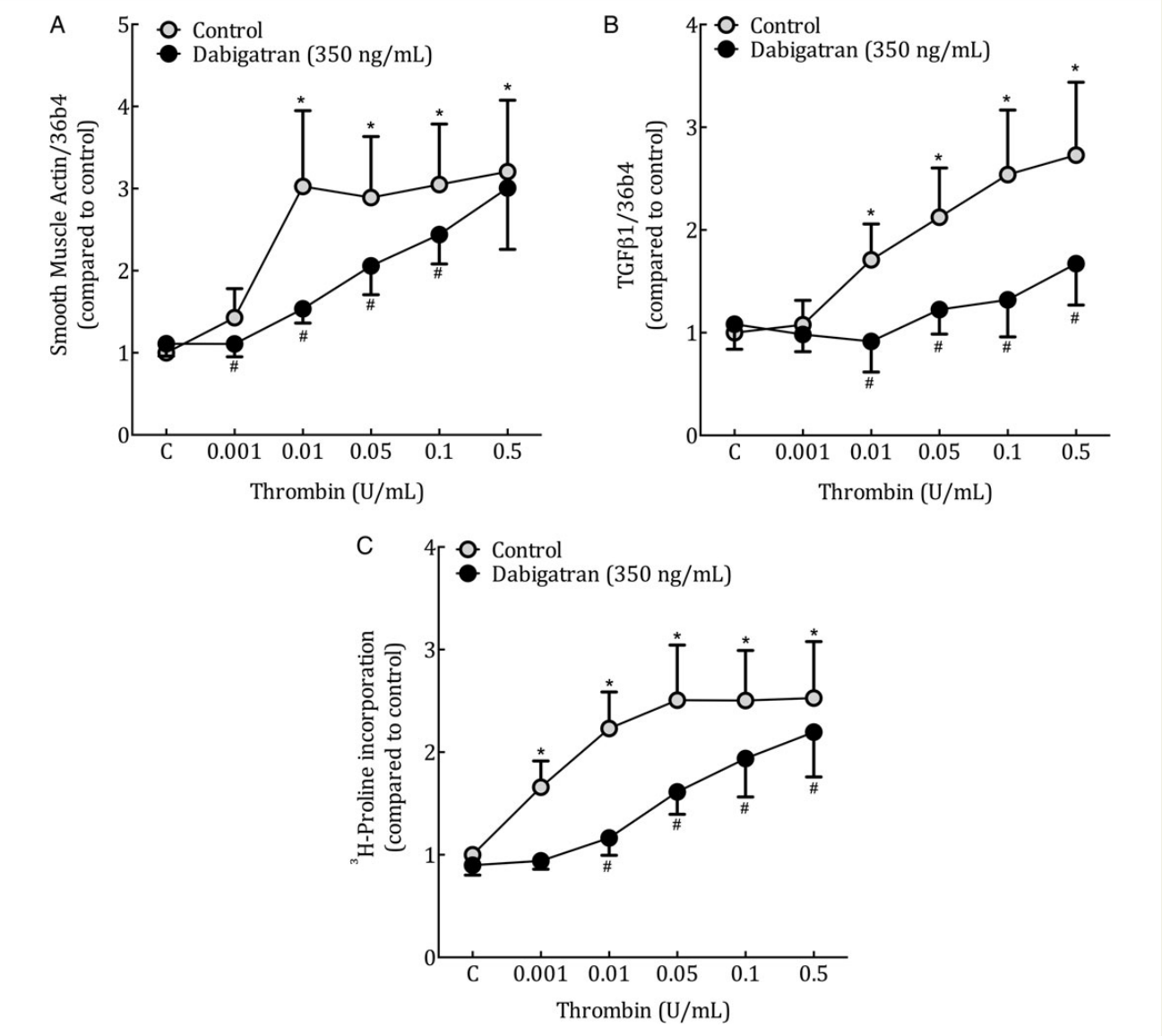

Figure 3 Thrombin increased expression of genes associated with differentiation towards myofibroblasts and fibrosis (*A*) α-smooth muscle actin ($n = 8–15$), (*B*) transforming growth factor β1 ($n = 8–15$), and increased incorporation of (*C*) ^{3}H-proline ($n = 4–13$). Dabigatran prevented these effects, except for the highest dosage of thrombin for α-smooth muscle actin and ^{3}H-proline. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with thrombin only.

associated with reduced complexity of the AF substrate, less endomysial fibrosis, and reduced αSMA staining in a well-characterized animal model of persistent AF. Taken together, these data suggest a role of hypercoagulability in the development of a substrate for AF.

Thrombin elicits pro-inflammatory and pro-fibrotic effects in atrial fibroblasts

This study demonstrates that in adult atrial fibroblasts, thrombin elicits pro-fibrotic and pro-inflammatory effects. These data are in line with other reports showing similar effects in fibroblasts isolated from other tissues, such as kidney and lung, as well as in ventricular cardiac fibroblasts, where a pro-fibrotic response upon stimulation with thrombin has been reported earlier.[29–32] Furthermore, our data suggest that this effect is mediated by activation of PAR1 as evidenced by the facts that PAR1 is the predominant isoform of PARs on fibroblasts, the fact that Trap-14, a PAR1 agonist, induced a response similar to the effect induced by thrombin, and that PAR1 blockade attenuated the increase in thrombin-induced ^{3}H-proline incorporation.

In vivo studies have demonstrated that PAR1-deficient mice show an attenuation of inflammation and fibrosis in a model of lung fibrosis.[33] Moreover, mice deficient in PAR1 performed better after ischaemia–reperfusion injury compared with their WT littermates, although PAR1 deficiency could not reduce infarct size.[12] PAR1 antagonists were also shown to

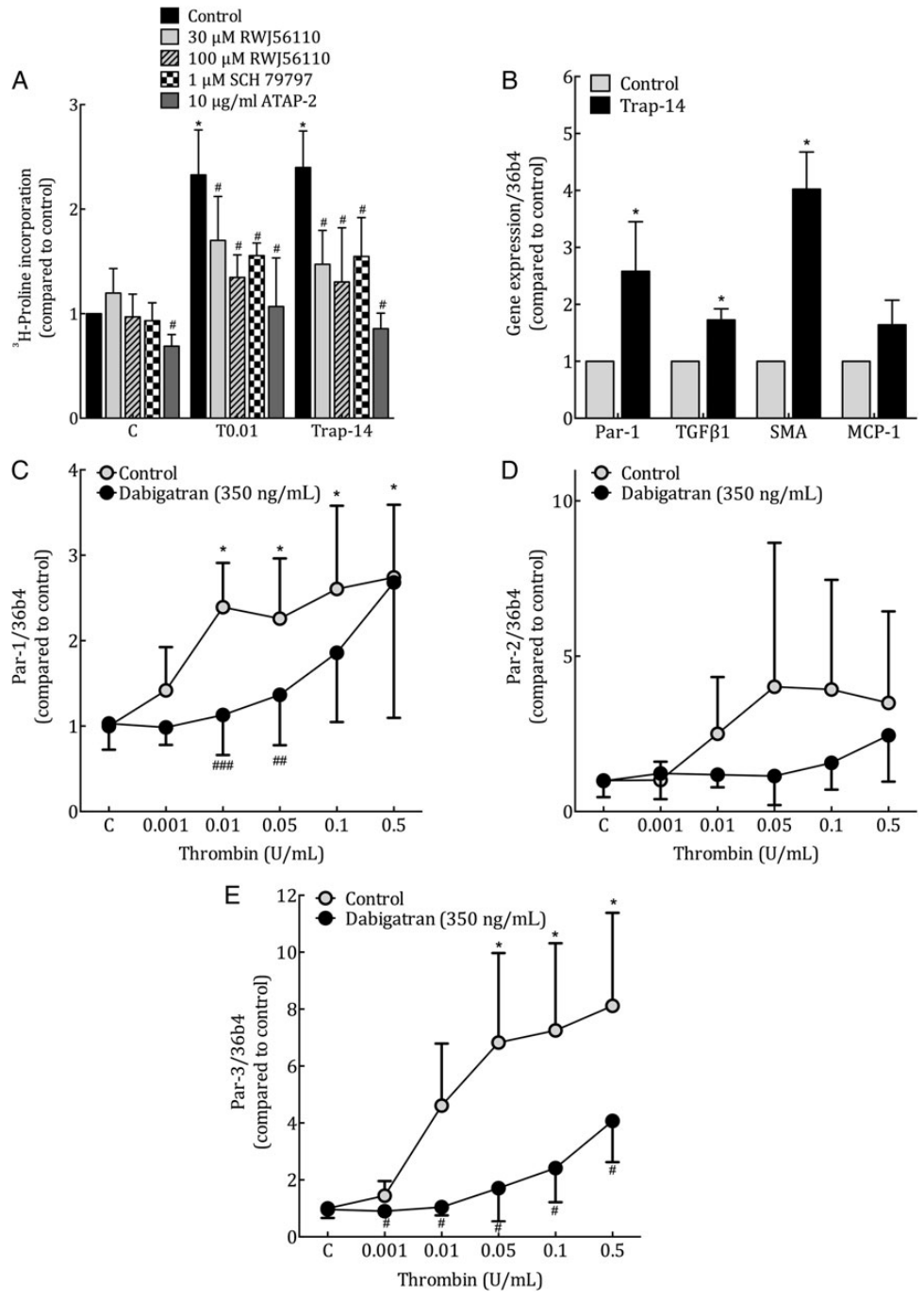

Figure 4 The pro-fibrotic effect of thrombin is mediated by protease-activated receptor-1. (*A*) ^{3}H-proline incorporation was reduced with protease-activated receptor-1-specific antagonists ($n = 2–7$). (*B*) 10 μM Trap-14 induced expression of pro-fibrotic genes ($n = 3$), (*C*) mRNA expression of protease-activated receptor-1 upon stimulation with thrombin ($n = 8–11$) and the effects of concomitant incubation with dabigatran. (*D*) mRNA expression of PAR2 upon stimulation with thrombin ($n = 4–6$) and the effects of concomitant incubation with dabigatran. (*E*) mRNA expression of PAR3 upon stimulation with thrombin ($n = 5–6$) and the effects of concomitant incubation with dabigatran. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with thrombin or Trap-14 only.

attenuate liver fibrosis and prevent LV remodelling upon ischaemia–reperfusion in rats.[15,31] In addition, PAR1 inhibition by SCH79797 reduced infarct size in an ischaemia–reperfusion model in rats.[34]

Earlier studies in neonatal fibroblasts suggested that thrombin may stimulate fibroblast proliferation, which potentially could contribute to tissue fibrosis.[11] Our study was performed in adult atrial fibroblasts and did not detect any effect of thrombin on fibroblast

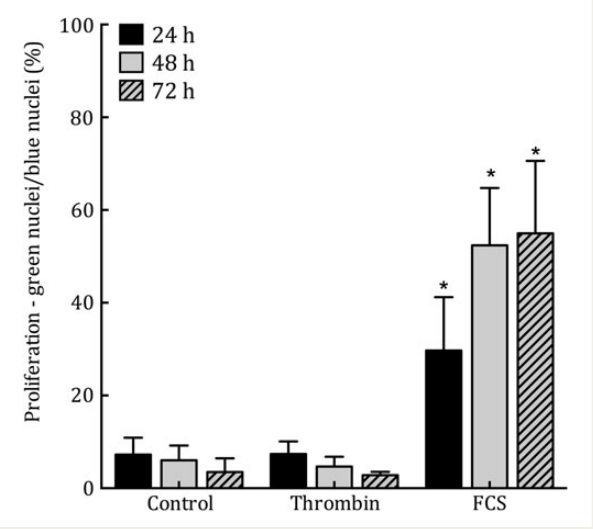

Figure 5 Thrombin did not induce fibroblast proliferation ($n = 3–7$). *$P < 0.001$ compared with control.

proliferation thereby confirming another study in adult cardiac fibroblasts that showed pro-fibrotic effects, but no effect of thrombin on cell proliferation.[32]

Hypercoagulability enhances atrial fibrillation susceptibility in transgenic mice

To test the hypothesis that the pro-fibrotic effect of thrombin activation would quantitatively be sufficient to produce a substrate for AF, we studied inducibility and stability of AF episodes in transgenic mice with a pro-coagulant phenotype. The $TM^{pro/pro}$ mice carry a mutation of the thrombomodulin gene leading to a reduced protein C-mediated degradation of Factors V and VIII. The resulting activation of the coagulation system has recently been demonstrated to aggravate the atherosclerotic phenotype in $ApoE^{-/-}$ mice.[20] Our data demonstrate that in these mice, inducibility and stability of AF as well as collagen deposition are enhanced and support the hypothesis that hypercoagulability can, under certain circumstances, produce a substrate for AF.

Nadroparin prevents the development of an atrial fibrillation substrate in goats

In goats with AF and a hypercoagulable state resulting from AF, inhibition of Factor Xa-mediated thrombin generation by nadroparin partially prevented the development of atrial fibrosis related to AF. Along with this, the complexity of AF and the number of αSMA-positive myofibroblasts were lower in the nadroparin-treated goats compared with the control animals. Together with the observed trend towards anticoagulant-induced diminished matrix degradation, as suggested by the reduced MMP-1/TIMP-1 ratio, these data strongly support the hypothesis not only that hypercoagulability produces atrial fibrosis during AF but also that inhibition of hypercoagulability may prevent the development of an AF substrate by partially preventing atrial fibrosis. Together with our notion of PAR1-dependent pro-fibrotic effects in atrial fibroblasts, these results suggest that the protective effect of nadroparin is due to the inhibition of PAR1-mediated pro-fibrotic activity of activated coagulation, as well as through reduced activity of proteins involved in extracellular matrix degradation. Absence of differences in TGFβ1 levels between controls and anticoagulated animals suggests that attenuation of atrial fibrosis through anticoagulation may be independent from the TGFβ1 pathway.

Potential implications for anticoagulant therapy

Until recently, patients with AF mostly received vitamin K antagonists (VKA) for stroke prevention. Vitamin K antagonists inhibit proper formation of vitamin K-dependent coagulation factors (prothrombin; Factors VII, IX, and X; protein C; and protein S). The proteins induced by vitamin K antagonism or absence (PIVKAs) occurring in patients on VKA therapy lack the Gla-domain, inhibiting binding to cell membranes thereby attenuating their pro-coagulant effects.[35,36] Interestingly, membrane binding does not seem necessary for activation of PARs by coagulation factors.[37] Therefore, the PIVKAs might still be able to activate PAR-mediated signalling, and patients on VKA are not protected against the pro-inflammatory and pro-fibrotic effects of PAR activation. As an alternative for VKA, non-VKA oral anticoagulants (NOACs) have become recommended for the prevention of stroke in patients with AF. Non-VKA oral anticoagulants directly inhibit thrombin (dabigatran) or Factor Xa (rivaroxaban, apixaban, edoxaban) and could possibly inhibit both coagulation and PAR activation. Therefore, NOACs might not only protect against stroke but may also have the potential to inhibit atrial fibrosis and the development of a substrate for AF. This hypothesis is further supported by the recent observation by Bukowska *et al.* who could demonstrate pro-inflammatory effects of activated Factor X on cultured human atrial tissue slices. In this study, pro-inflammatory signalling and up-regulation of the adhesion molecule ICAM-1 were prevented by pre-exposure of the tissue slices to the Factor X antagonist rivaroxaban.[38] To the best of the authors' knowledge, clinical studies to test the hypothesis that NOACs but not VKAs delay the process of structural remodelling in the atria and the progression of AF have not been undertaken yet. It remains to be determined whether in patients NOACs can delay the progression of AF. Whether confirmation of this hypothesis would lead to a broadening of the indication for anticoagulation to patients with a CHA_2DS_2-VASc score of one is questionable, given the bleeding risk associated with anticoagulant compounds. However, the results of our study demonstrate that PAR inhibition independently from anticoagulant activity is a promising target for the development of new upstream therapies for AF.

Conclusion

In summary, thrombin induced a pro-fibrotic and pro-inflammatory response in adult atrial fibroblasts by activation of the PAR1 receptors, suggesting a potential role of activated coagulation in atrial remodelling and the development of a substrate for AF. Also, hypercoagulability enhanced susceptibility to AF in a transgenic mouse

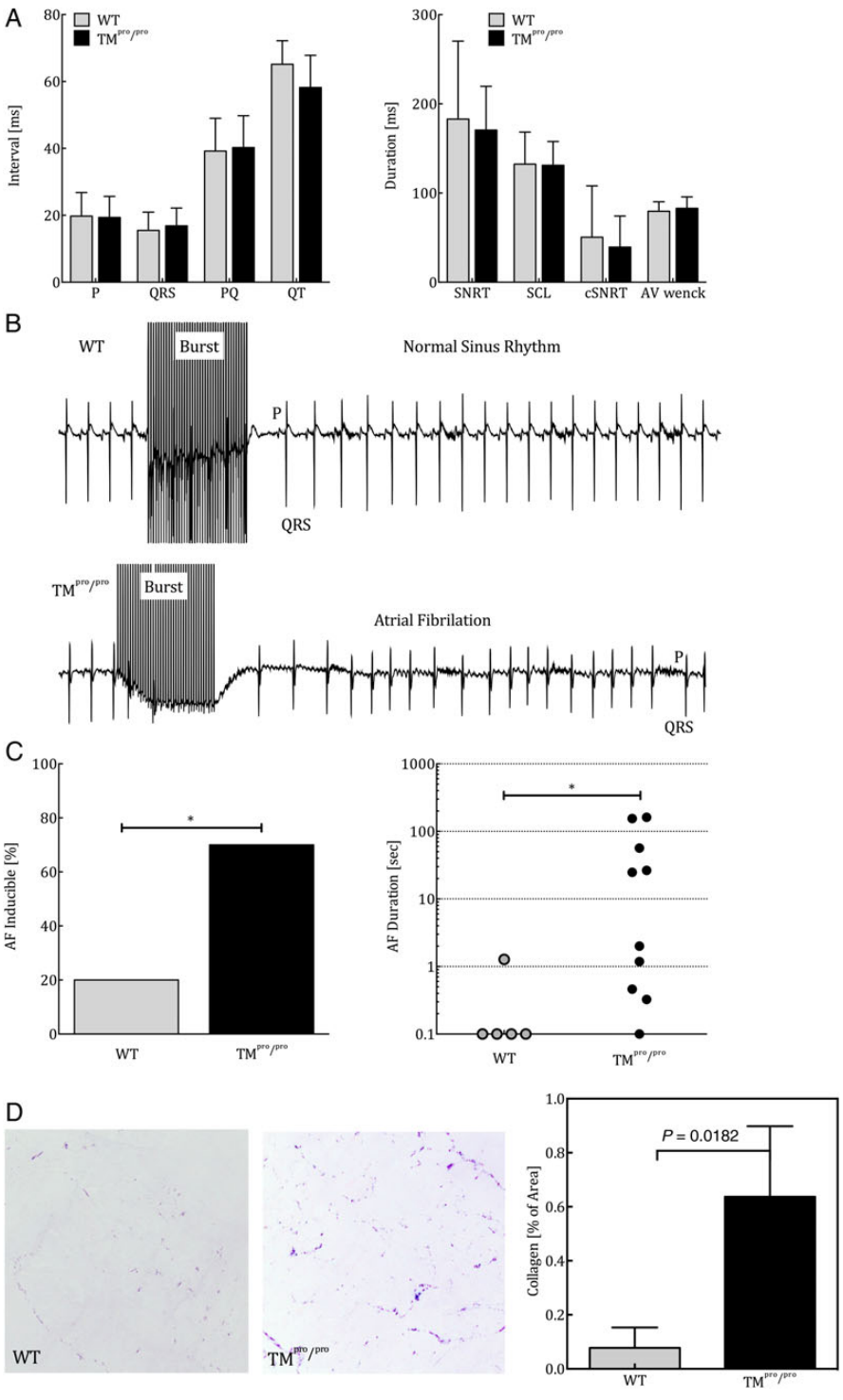

Figure 6 Cardiac electrophysiology in mice with a hypercoagulable phenotype. (*A*) Right panel: Electrocardiogram intervals in wild-type and $TM^{pro/pro}$ mice (duration of the P wave, QRS duration, PQ and QT intervals). Left panel: sinus node recovery time, sinus cycle length, corrected sinus node recovery time (= sinus node recovery time − sinus cycle length), and AV Wenckebach cycle length. (*B*) Transoesophageal burst pacing in a wild-type and $TM^{pro/pro}$ mouse. In the latter, burst pacing induced an episode of atrial fibrillation, whereas the wild-type mouse returned to sinus rhythm immediately after the burst. (*C*) Inducibility of AF (episode lasting >1 s) by burst pacing (left panel) and AF episode durations (right panel). In a significantly higher proportion of $TM^{pro/pro}$ mice (7 out of 12 vs. 1 out of 10), atrial fibrillation could be induced, and atrial fibrillation episodes were significantly longer. (*D*) Enhanced collagen deposition (Sirius red staining) in heart atria of $TM^{pro/pro}$ mouse (0.457% ± 0.114) compared with wild-type animals (0.078% ± 0.025, $P = 0.0182$). The left picture shows a typical example of wild-type atria and the right picture of an atria from a $TM^{pro/pro}$ mouse. *$P < 0.05$.

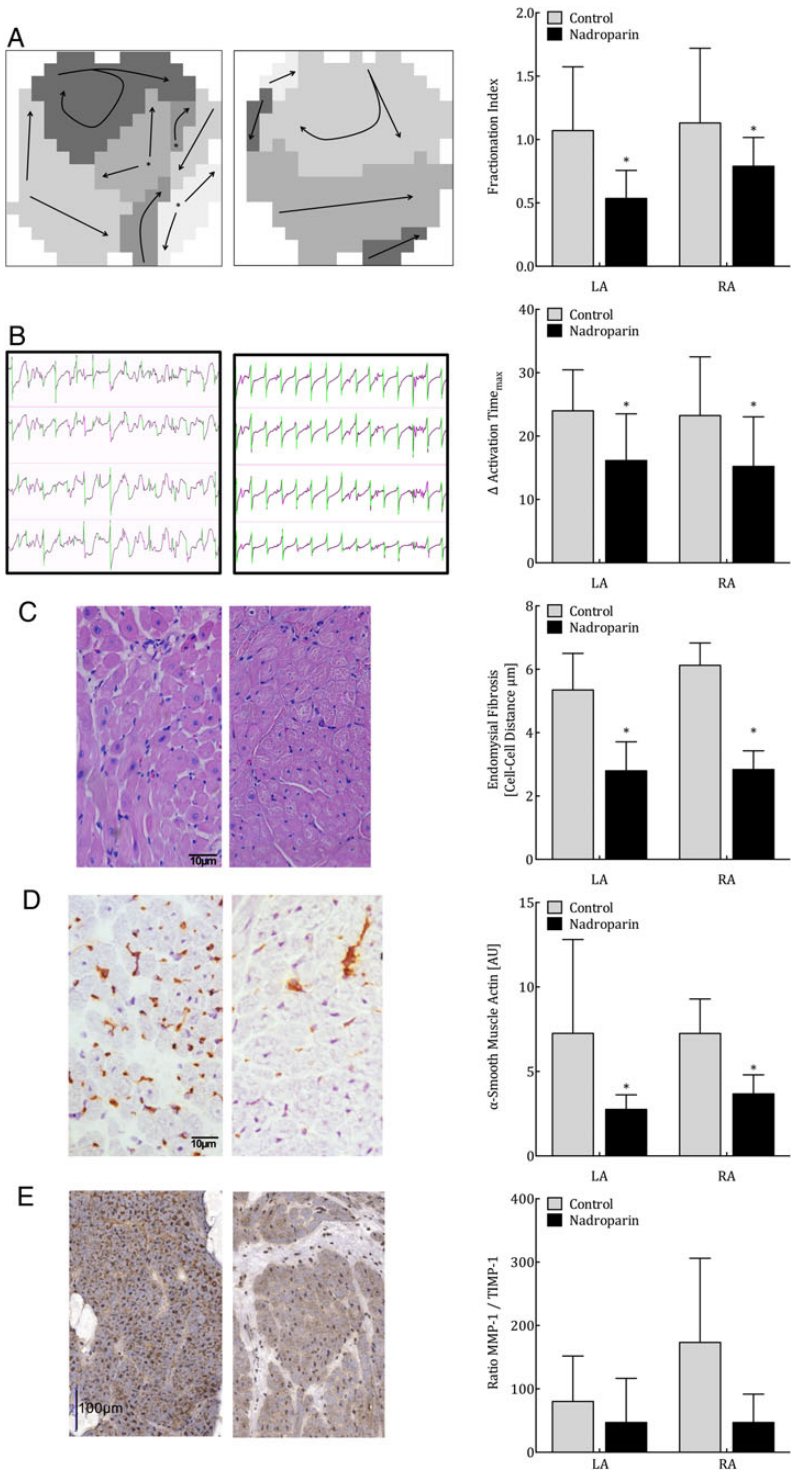

Figure 7 Effect of nadroparin on the development of a substrate for atrial fibrillation. (*A*) Representative waves maps during atrial fibrillation in the LA of a control goat (left) and a goat treated with nadroparin (mid panel). Complexity of atrial fibrillation quantified as maximal activation times differences per atrial fibrillation beat was significantly lower in both LA and RA of goats treated with nadroparin ($n = 6$) and control ($n = 9$). (*B*) Representative fibrillation electrograms. The unipolar fractionation index was lower in the treated compared with the untreated animals. (*C*) Endomysial fibrosis quantified as cell–cell distances in the epicardial layer of the atrial wall. In the nadroparin-treated group, less atrial fibrillation-related atrial fibrosis was present. (*D*) Nadroparin-treated animals showed reduced numbers of α-smooth muscle actin-positive myofibroblasts (right staining panel) compared with controls (left panel). (*E*) A trend towards a reduced matrix metalloproteinase-1/ tissue inhibitor of matrix metalloproteinase-1 ratio in an animals treated with nadroparin ($P = 0.0212$ for difference between combined left and right atria for untreated vs. treated). Representative immunohistochemical staining for matrix metalloproteinase-1 in the right atria of a control (left) and an anticoagulated (right) goat. *$P < 0.05$ nadroparin vs. control.

Table 2 Effect of nadroparin treatment on atrial fibrillation cycle length

	Control	Nadroparin	*P*-value
AFCL (ms)			
RA	125 ± 29	113 ± 37	0.472
LA	135 ± 25	125 ± 21	0.401

Data presented as mean ± SD for LA and RA of goats treated with nadroparin ($n = 6$) and control ($n = 9$).

model. Atrial fibrillation goats treated with nadroparin, which lowers Factor Xa-mediated thrombin generation and thus PAR activation, showed lower AF complexity and less AF-related fibrosis. These observations are particularly interesting as NOACs have been demonstrated to effectively inhibit PAR stimulation. Future studies will have to address the question whether hypercoagulability is also associated with progression of atrial remodelling in patents with AF and whether anticoagulants can also effectively prevent atrial fibrosis in these patients.

Limitations

Fibroblasts in culture differentiate rapidly into myofibroblasts.[39,40] For this reason, we used fibroblasts from a low passage. As expected, a large part of our cells expressed smooth muscle actin, suggesting the myofibroblasts phenotype. Nevertheless, thrombin exposure could clearly accentuate the myofibroblast phenotype even further and provoke pro-inflammatory and pro-fibrotic responses.

We used nadroparin treatment in the goats as its effect on the coagulation system is well known and can accurately be monitored. The use of dabigatran for direct thrombin inhibition in the *in vitro* model and nadroparin for inhibition of both thrombin and Factor Xa in the *in vivo* goat model can be considered as a limitation of the study. Since the pharmacodynamics of direct thrombin or Factor Xa inhibitors in goats are not fully known, we used parenteral inhibition of coagulation with low molecular weight heparin. Also, the protective effect of NOACs in other paradigms of AF, e.g. heart failure-induced atrial remodelling, should be addressed. Further studies will have to establish an effect of NOACs on the atrial remodelling process in animal models of different types of AF and in patients with AF or with a high susceptibility to AF.

Authors' contributions

H.M.H.S., A.M.D.J., S.V., H.C.D.B., A.H.M., D.H.L., M.R., P.W.K., H.t.C., H.C., I.C.V.G., A.J.v.Z., and U.S. performed statistical analysis. H.M.H.S., A.M.D.J., S.V., P.W.K., H.t.C., H.C., I.C.V.G., A.J.v.Z., and U.S. handled funding and supervision. H.M.H.S., A.M.D.J., S.V., H.C.D.B., A.H.M., D.H.L., M.R., A.v.H., M.K., S.L., S.Z., D.L., P.W.K., H.t.C., H.C., I. C.V.G., A.J.v.Z., and U.S. acquired the data. H.M.H.S., A.M.D.J., S.V., H.C.D.B., A.H.M., P.W.K., H.t.C, H.C., I.C.V.G., A.J.v.Z., and U.S. conceived and designed the research. H.M.H.S., A.M.D.J., S.V., H.C.D.B., A.H.M., D.H.L., M.R., A.v.H., M.K., S.L., S.Z., D.L., P.W.K., H.t.C., H.C., I.C.V.G., A.J.v.Z., and U.S. drafted the manuscript. H.M.H.S., A.M.D.J., S.V., H.C.D.B., A.H.M., D.H.L., M.R., A.v.H., M.K., S.L., S.Z., D.L., P.W.K., H.t.C., H.C., I.C.V.G., A.J.v.Z., and U.S. made critical revision of the manuscript for key intellectual content.

Acknowledgement

The expert technical assistance for the immunolabeling provided by A.M. van Oeveren-Rietdijk, MSc is greatly acknowledged.

Funding

This work was supported by the Netherlands Heart Foundation (CVON2014-09, RACE V, Reappraisal of Atrial Fibrillation: Interaction between hyperCoagulability, Electrical remodeling, and Vascular Destabilisation in the Progression of AF) to U.S., H.M.H.S., H.t.C., A.J.v.Z., H.J.C., P.W.K., M.R., and I.C.V.G. The work was furthermore supported by the European Union (FP7 large collaborative project EUTRAF, No. 261057, the Horizon2020 project CATCH-ME: Characterizing Atrial fibrillation by Translating its Causes into Health Modifiers in the Elderly, No. 633196, the ITN Network AFib TrainNet, No. 675351, the ITN Network RADOX: RADical reduction of OXidative stress in cardiovascular diseases, PITN-GA-2012-316738) to U.S., S.V., and H.J.C. This work was furthermore supported by a grant from the Interuniversity Cardiology Institute Netherlands (to A.M.D.J.) and a grant from the Netherlands Organisation of Scientific Research (NWO, Veni grant 016.136.055 to M.R.).

Conflict of interest: H.t.C. is a Fellow of the Gutenberg Research College, Gutenberg University, Mainz, Germany.

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