Molecular and cellular pharmacology

Coagulation factor Xa induces an inflammatory signalling by activation of protease-activated receptors in human atrial tissue

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ABSTRACT

Activated factor X (FXa) is an important player in the coagulation cascade responsible for thrombin generation, which is activated during atrial fibrillation. Increasing evidence suggests that FXa influences cell signalling in various cell types by activating protease-activated receptors (PARs). It is so far not known if molecular effects of FXa affect atrial signal transduction. To study the effects of FXa, human atrial tissue slices were cultivated with FXa up to 24 h. Additionally, rapid pacing was applied at 4 Hz to resemble atrial fibrillation. The inhibitory impact of FXa antagonist (Rivaroxaban), protease-activated receptor 1 antagonist (SCH79797), and protease-activated receptor 2 antagonist (GB83) were analysed under experimental conditions. The exposure of atrial tissue to FXa resulted in the 1.7 fold upregulation of PAR2-mRNA, activation of MAP kinases (ERK1/2) and NF-κB signalling. Furthermore FXa increased the expression of adhesion molecule ICAM-1 (1.82 ± 0.20), chemokine IL-8 (1.94 ± 0.20), as well as prothrombotic molecule PAI-1 (1.52 ± 0.17). The combination of rapid pacing and FXa caused significant upregulation of PAR1 (2.82 ± 0.22), PAR2 (2.66 ± 0.40), ICAM-1 (2.13 ± 0.25), IL-8 (2.22 ± 0.24), LOX-1 (2.59 ± 0.35), and PAI-1 (2.65 ± 0.52) at the mRNA level. Rivaroxaban and GB83 prevented upregulation of PARs, ICAM-1, LOX-1, IL-8, and activation of MAP kinases. The elevation in the expression of PAI-1 was hindered in the presence of SCH79797, or Rivaroxaban. The present study indicates that FXa mediates inflammatory signalling in atrial tissue. Importantly, FXa and tachyarrhythmia act synergistically to increase expression of protease-activated receptors and inflammatory mediators. Rivaroxaban prevented effectively FXa-induced molecular effects in human atrial tissue particularly during rapid pacing.

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1. Introduction

Atrial fibrillation is the most common cardiac arrhythmia. Its incidence and prevalence constantly rise with age. Atrial fibrillation not only impairs quality of life, but also increases mortality due to its attributed stroke risk and heart failure (Schotten et al., 2011). Atrial fibrillation is a major contributor to ischaemic stroke at all ages (about 30%) due to thrombus formation and embolisation from the left atrial appendage (Lip, 2011; Watson et al., 2009). Depending on clinical risk factors like hypertension, age, diabetes

mellitus etc., the individual risk for thromboembolic events varies from about 2% to 18% per year (Lip, 2011). Recent studies showed the importance of prothrombotic endocardial changes for the development of atrial thrombi (Schotten et al., 2011; Watson et al., 2009; Goette et al., 2008). The initiating mechanism appears to be an oxidative stress-induced elevated expression of adhesion molecules, plasminogen-activator inhibitor-1 (PAI-1), and von-Willebrandt factor (vWF), which allows endocardial recruitment of inflammatory cells and leukocyte-platelet conjugates to the endothelium of fibrillating atria (Kamiyama, 1998; Hammwöhner et al., 2007; Bukowska et al., 2008; Dudley et al., 2005). Thus, therapeutic interventions, which may interfere with these cellular signalling processes, might be beneficial in patients with atrial fibrillation.

The activated coagulation factor X (FXa) is a serine protease and plays a central role in the coagulation cascade linking the extrinsic and intrinsic pathway. During haemostasis, FXa associated with FVa and phospholipids forms the prothrombinase complex and activates prothrombin to thrombin. In addition to its important

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Table 1
The clinical characteristic of patients.

Patients	$n = 54$
Gender M/F	38/16 [70%/30%]
Age	69 ± 9
Body mass index in kg/m^3	29 ± 4
Hypertension	45 [83%]
Diabetes mellitus	23 [43%]
Hyperlipoproteinemia	43 [80%]
Left ventricular hypertrophy	39 [72%]
Left ventricular ejection fraction	$51 \pm 9\%$
Previous myocardial infarction	27 [50%]
Coronary heart disease	2.7 ± 0.6
NYHA classification	2.3 ± 0.6
Previous thromboembolism/stroke	7 [13%]
History of smoking	31 [57%]
Current smoking	3 [5%]
Therapy	
ACE inhibitors	26 [48%]
Angiotensin II receptor antagonists	21 [39%]
Statins	35 [65%]
Beta blockers	36 [67%]
Ca-antagonists	16 [30%]
Nitrate	15 [28%]
Anticoagulants	38 [70%]
Diuretic	25 [46%]
ASA	43 [80%]

Values are mean $\pm$ SD or n [%]; percentages are calculated according to available data set. F, female, M, male. ACE, angiotensin-converting enzyme; ASA, acetylsalicylic acid; NYHA, New York Heart Association-Classification.

role in the coagulation cascade, increasing evidence shows that FXa acts as a signalling molecule mediating cellular responses by activating protease-activated receptors (PARs) (Steinberg, 2005; Ossovskaya and Bunnet, 2004). Receptor desensitisation studies with peptide agonists (PAR1 or PAR2) and experiments with protease-activated receptor 1 – blocking antibodies indicate that FXa-signalling is mediated by both protease-activated receptors (McLean et al., 2001).

The activation of protease-activated receptors influences a broad range of intracellular pathways, e.g. stimulation of phospholipase C, mobilisation of calcium ions (Bohm et al., 1996; Shapiro et al., 2000), suggesting that these receptors are capable of activating Gq signalling. The G protein-coupled receptors tend to interact within the subtypes. Thus, the response to activation of protease-activated receptors depends on the G protein couple formation and effectors repertoire expressed in the cell type (Trejo, 2003; Macfarlane et al., 2001).

So far, the effect of FXa and its combined effect with rapid tachyarrhythmia on cellular signal transduction have not been studied in human atrial tissue. The FXa-related outside-in signalling might be of particular importance in stressed tissues like fibrillating atrial myocardium. Thereby, FXa could be an additional factor causing the well-described phenomenon of structural atrial remodelling in patients with atrial fibrillation.

2. Materials and methods

2.1. Heart tissue samples

Right atrial appendages were obtained from 54 patients undergoing heart surgery for coronary artery bypass graft or mitral/aortic valve replacement. None had a history of documented sustained arrhythmias. Treatments were usually stopped 24 h before surgery. The clinical characteristics are shown in Table 1. The study conforms to the Declaration of Helsinki and was approved by the local Ethics Committee. All patients gave written informed consent to participate in the study.

2.2. Culturing tissue slices and "in vitro" pacing

The atrial tissue slices were prepared in cold, oxygenated (95% O$_2$/5% CO$_2$) Tyrode buffer using a vibratome (VT1200S; Leica Microsystems, Germany). The 350 μm slices were placed on top of membrane of tissue inserts (0.4 μm Millipore, Germany) and cultivated in a Petri dish, which was filled with serum-free DMEM medium (Invitrogen, Germany) implemented with 100 nM of hirudin (Refludan, Pharmion Germany) for up to 24 h in an incubator (21% O$_2$, 5% CO$_2$, 37 °C).

For stimulation in an electrical field, a pair of custom-built carbon electrodes was submersed at the opposite ends of a Petri dish and connected to a stimulation unit (GRASS Stimulator) as previously described (Bukowska et al., 2006). Pacing of the tissue slices was performed within the serum-free DMEM medium up to 24 h at 37 °C at rates of 0.6 and 4.0 Hz. An antagonist of FXa, Rivaroxaban (provided by Bayer HealthCare, Germany), antagonists of protease-activated receptor 1 (SCH79797 hydrochloride), and 2 (GB83) (Axon Medchem, Groningen, Netherlands) were added to the medium 1 h before the exposure to FXa or the stimulation in the electrical field. The activated human factor X (FXa) was used in the concentration of 50 nM (Dunn, Germany). The viability of human atrial tissue slices cultured or stimulated in vivo was confirmed using established vital staining technique (CellTracker Green CMFDA, Invitrogen, Karlsruhe, Germany) (Bussek et al., 2009). The living slices were incubated for 1–2 h with the final concentration of 5 μM dye in serum-free medium and imaged as stitched mosaic with a fluorescent microscope (Zeiss Axiovert 200 m) using an excitation wavelength filter of 480/40 nm, a diachroic filter 505 nm long pass and emission wavelength between filter of 535/50 nm. The cellular integrity of tissue slices was assessed using lactate dehydrogenase (LDH) assay (Roche Diagnostics, Mannheim, Germany). The enzyme release from slices into culture medium was measured after 24 h of incubation. To determine the total LDH content, atrial slices were collected, homogenised in 1 ml medium with 0.5% triton X-100. The test was performed according to the manufacturer's instructions.

2.3. Quantitative RT-PCR

Total RNA was isolated from atrial tissue slices using Nucleo SpinRNAII (Macherey-Nagel, Germany) in combination with TRIzol/chloroform extraction (Invitrogen, Germany). Reverse transcription was carried out with 1 μg of each RNA using random primer with Fermentas kit according to the manufacture's protocol. (Fermentas, Germany). Quantitative PCR was performed using the iCycler (BioRad, Munich, Germany). A 25 μl reaction mixture consisted of $1 \times$ SensiMix (Quantace, Bioline, UK), 1 μl cDNA, and 0.3 μM of the specific primers (Table 2). Initial denaturation at 95 °C for 600 s was followed by 40 cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 20 s. The expression analyses were carried out according to the ΔΔCT method. Amplification specificity of PCR products was confirmed by melting curve analysis and agarose gel electrophoresis.

2.4. Western blot analysis

Atrial tissue slices were homogenised in modified RIPA buffer, which contained 50 mM Tris–HCl (pH 7.5), 100 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 10% glycerol, 10 mM potassium dihydrogen orthophoshate, 0.5% nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 0.5% deoxylate, 20 mM sodium fluoride, 20 mM glycerol-2-phosphate, and a protease inhibitor cocktail (all from Sigma, Heidelberg, Germany).

Table 2
Primer sequences used for real-time PCR.

Gene	GenBank accession number	Primer sequence	
PAR1	M62424	Forward	AAGTCAGGAGAGAGGGTGAAGC
		Reverse	GATCTAAGGTGGCATTTGTTGC
PAR2	NM_005254	Forward	CACCATCCAAGGAACCAATAG
		Reverse	AATTGGAAGGAAGACAGTGGTC
ICAM-1	BC015969.2	Forward	AGTCAGTGTGACCGCAGAGG
		Reverse	CCTGGCACATTGGAGTCTGC
IL-8	NM_000584.3	Forward	AGATGTCAGTGCATAAAGACA
		Reverse	TATGAATTCTCAGCCCTCTTCAAAAA
LOX-1	AF035776	Forward	ACAGATCTCAGCCCGGCAACAAGCA
		Reverse	GGGAGACAGCCGCCTCGGACTCTAAAT
PAI-1	NM_000602	Forward	ACTGGAAAGGCAACATGACC
		Reverse	TGACAGCTCTGGATGAGGAG
TM	BC035602	Forward	CAGAGCCAACTGCGAGTACC
		Reverse	CACAGTCGGTGCCAATGTGG
ß-actin	NM_001101	Forward	AAGATGACCCAGATCATGTTTGAG
		Reverse	AGGAGGAGCAATGATCTTGATCTT

Tissue homogenates were centrifuged at 16,000g for 30 min and the resulting supernatant (total tissue homogenate) was stored at −20 °C for analysis. Protein samples of 20 μg each were separated by SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibody against phospho-44/42 ERK (1:1000; Cell Signaling, MA, USA), 44/42ERK (1:1000, Cell Signaling, MA, USA), phospho-NF-κBp65 (1:1000; Cell Signaling, MA, USA), ICAM-1 (1:1000, Santa Cruz, USA), PAI-1 (1:300, Santa Cruz, USA), LOX-1(1:1000, R&D Systems, Germany), PAR2 (1:500, Santa Cruz, USA), ß-actin (1:5000, Santa Cruz, USA), followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody and detection using enhanced chemiluminescence (Pierce, Rockford, USA). The protein expression was quantified using AlphaEaseFC software (AlphaImager System, Alpha Innotech, CA, USA).

2.5. Statistical analysis

All values are expressed as mean ± S.E.M. if not indicated otherwise. Statistical analyses were performed using Origin 8.5 (OriginLab Corporation, USA). A *P* value of < 0.05 was considered to be statistically significant (ANOVA).

3. Results

3.1. Viability of human atrial tissue slices

To study the impact of activated coagulation factor X (FXa) on human atrial tissue, slices from right appendages were prepared and cultured up to 24 h. The viability of the culturing or stimulated in the electrical field human slices is demonstrated in Fig. 1. Moreover, the cellular integrity of atrial slices was monitored by measurements of lactate dehydrogenase (LDH) under different study conditions. The LDH leakage during culturing experiments or stimulation in the electrical field was determined as a percentage of the total LDH present in the slices as shown in Fig. 1.

3.2. Activation of ERKs in atrial tissue

Research on the signalling mediated by coagulation factors in endothelial cells has shown that thrombin and FXa activate mitogen-activated protein kinases. In a first set of experiments, we analysed the phosphorylation state of ERK kinases in atrial tissue caused by different concentrations of FXa (Fig. 2A). To

maximise the signal in the presence of 100 nM hirudin, which inhibits possible effects of thrombin, we decided to use FXa in the concentration of 50 nM. As shown in Fig. 2B, the concentration of 1 μM of Rivaroxaban was necessary to attenuate the FXa-dependent activation of ERK under experimental conditions.

We analysed the phosphorylation state of ERK kinases in atrial tissue after 2 h of stimulation with FXa and followed up to 24 h. Fig. 2C illustrated the sustained character of FXa-dependent activation of ERK kinases, which was diminished effectively by Rivaroxaban even after 24 h of exposure to FXa. To approach the condition of atrial fibrillation, we stimulated atrial tissue slices in the electrical field with the frequency of 4 Hz in the presence of FXa. Under different experimental conditions, GB83, an antagonist of protease-activated receptor 2, and Rivaroxaban, an antagonist of FXa, showed similar inhibitory effects on ERK activation (Fig. 2C, D). An antagonist of protease activated receptor 1, SCH79797 significantly prevented the activation of p44-ERK (ERK2).

3.3. Protease-activated receptors in response to FXa and tachypacing in human atrial tissue

Coagulation factors mediate their action through protease-activated receptors (PARs). To investigate which of the isoforms contribute to the FXa-dependent response in atrial tissue, we exposed atrial slices to FXa for 24 h and analysed changes in the expression of protease-activated receptor 1 and 2. As illustrated in Fig. 3B and E, FXa enhanced the expression of protease-activated receptor 2 at the transcriptional and at the protein level. In addition, tachypacing and FXa acted synergistically on the expression of protease-activated receptor 2. However, the combined stimulation in the electrical field with presence of FXa increased the expression of protease-activated receptor 1 considerably more than pacing alone (Fig. 3A). Rivaroxaban achieved very similar effects to protease-activated receptor 2 antagonist (GB83) in preventing the FXa-dependent increase of the protease-activated receptor 2 expression (Fig. 3B, C). Interestingly, both antagonists significantly counteracted the tachypacing-dependent induction of protease-activated receptor 1 expression. In contrast, protease-activated receptor 1 antagonist, SCH79797 showed high specificity toward protease-activated receptor 1 and influenced the expression of protease-activated receptor 2 to a lesser extent.

3.4. Activation of NF-κB signalling in atrial tissue

Different inflammatory and oxidative effectors cause activation of kinases, which, in consequence, phosphorylate the transcription factor subunits, NF-κBp65 and NF-κBp50. The phosphorylation at Ser-536 of NF-κBp65 enables the release from the inhibitory complex and moving into the nucleus. In our work we decided to follow this phosphorylation state to evaluate the activation of NF-κB signalling after exposure to FXa. As shown in Fig. 4A, Western Blotting analyses revealed the sustained character of the activation of NF-κBp65 in atrial tissue. The combined stimulation for 24 h showed a significant increase in the phosphorylation at Ser536 of NF-κBp65 (Fig. 4A lower panel).

Whereas Rivaroxaban achieved its highest inhibitory effects toward the NF-κB activation after 2 h exposure to FXa, GB83 presented prolonged activity (Fig. 3A, B). The antagonist of protease-activated receptor 1 affected the NF-κB activation to a lesser extent.

3.5. Induction of NF-κB target protein in atrial tissue

The transcription factor NF-κB regulates expression of various inflammatory, prooxidative, and prothrombotic molecules. In this work, we drew our attention to molecules participating in the atrial thrombogenicity such as an intracellular adhesion molecule
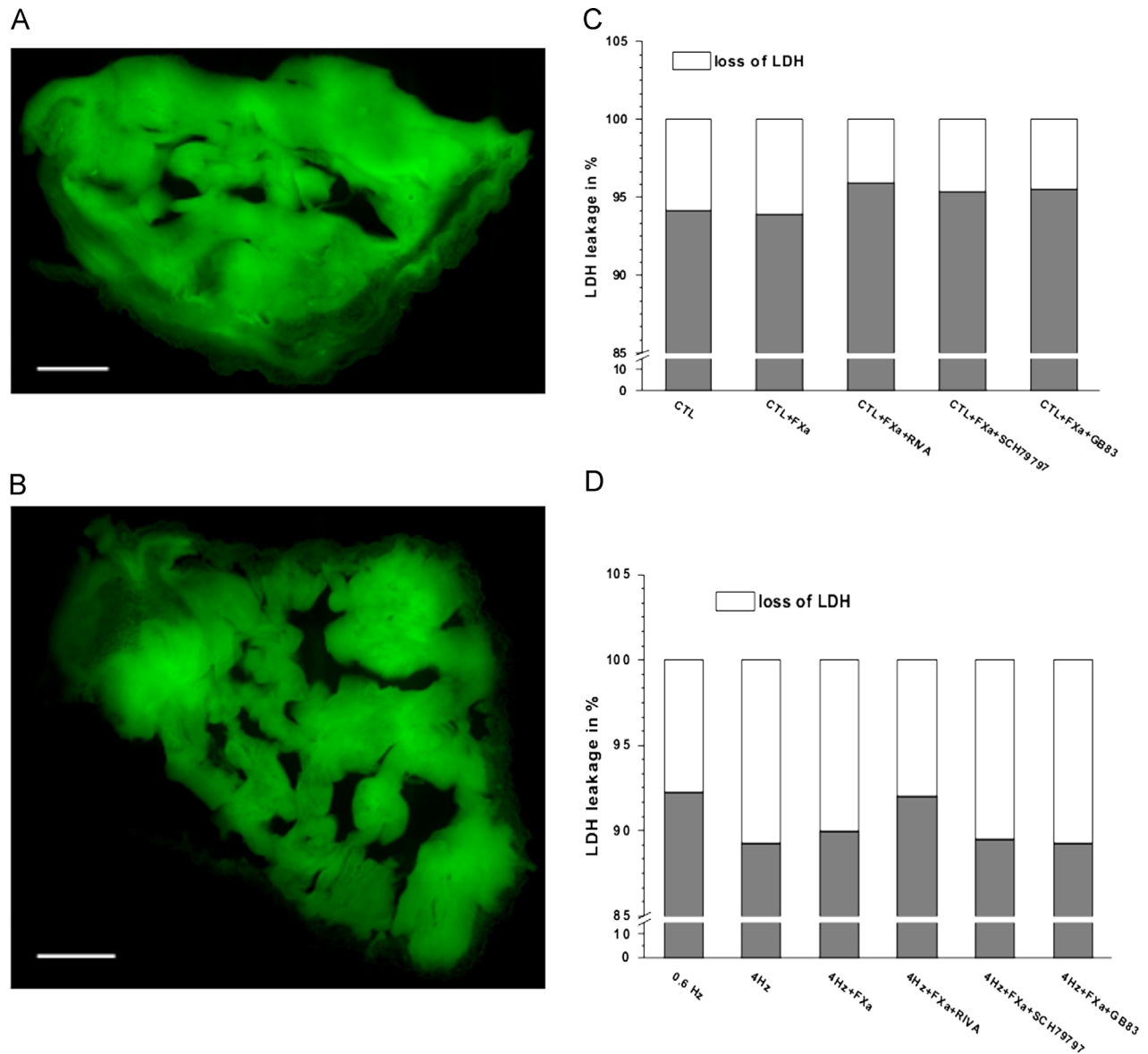
Fig. 1. Viability of tissue slices. Representative stitched image of 350 μm thick human slices from right appendages stained with CellTracker green after 24 h of culturing (A), and after 24 h stimulation in the electrical field (B). The integrity of atrial slices was expressed by LDH leakage during culturing (C) or stimulation in the electrical field. (D) The LDH leakage was determined as a percentage of the total LDH present in the slices. Results are mean ± standard deviation at least 4 experiments. Scale bar: 1000 μm.

(ICAM-1), interleukin-8 (IL-8), plasminogen activator inhibitor (PAI-1), and receptor for oxidised LDL (LOX-1).

The expression of ICAM-1 was upregulated in response to FXa at transcriptional and protein level (Fig. 4A, C, D). The additional exposure of atrial tissue slices to 4 Hz in an electrical field resulted in a significant increase in ICAM mRNA and protein contents. GB83 offered the highest inhibitory effect on ICAM induction even after 24 h of exposure to FXa. The protective effects of Rivaroxaban were highest after 2 h exposure to FXa and lowered with time particularly under resting condition. SCH79797 affected the ICAM-expression to a less extent.

Atrial tissue slices responded to FXa with a significantly increase in IL-8 transcriptional level. The rapid pacing acted synergistically on IL-8 expression. All applied antagonists prevented the induction of IL-8 mRNA significantly (Fig. 4E).

The expression of LOX-1, a prooxidative marker, remained unchanged after exposure to FXa (Fig. 5). However, this expression was increased 2.5-fold on the mRNA and protein level with addition of rapid pacing. Interestingly, Rivaroxaban as well as antagonists of

protease-activated receptors 1 and 2 significantly prevented upregulation in the LOX-1 expression.

The exposure of atrial tissue slices to FXa for 24 h as well as to stimulation in the electrical field affected the expression of prothrombotic molecules, PAI-1 at mRNA and protein level (Fig. 6). The combination of rapid electrical stimulation with administration of FXa synergistically increased the expression of PAI-1 (Fig. 6B, C). Interestingly, the antagonist of protease-activated receptor SCH79797 and Rivaroxaban offered the highest inhibitory effects on PAI-1 expression under all experimental conditions. In this case, antagonist of protease-activated receptors 2 prevented the FXa-induced expression of PAI-1 to a lesser degree. The inhibitory effects of the specific antagonist of protease-activated receptor 1 on PAI-1 expression strong imply an involvement of this receptor in mediation of the FXa-signalling.

In contrast to PAI-1, the expression anti-thrombotic molecule thrombomodulin was neither affected by FXa nor by rapid pacing. Rivaroxaban had no effect on thrombomodulin (data not shown).
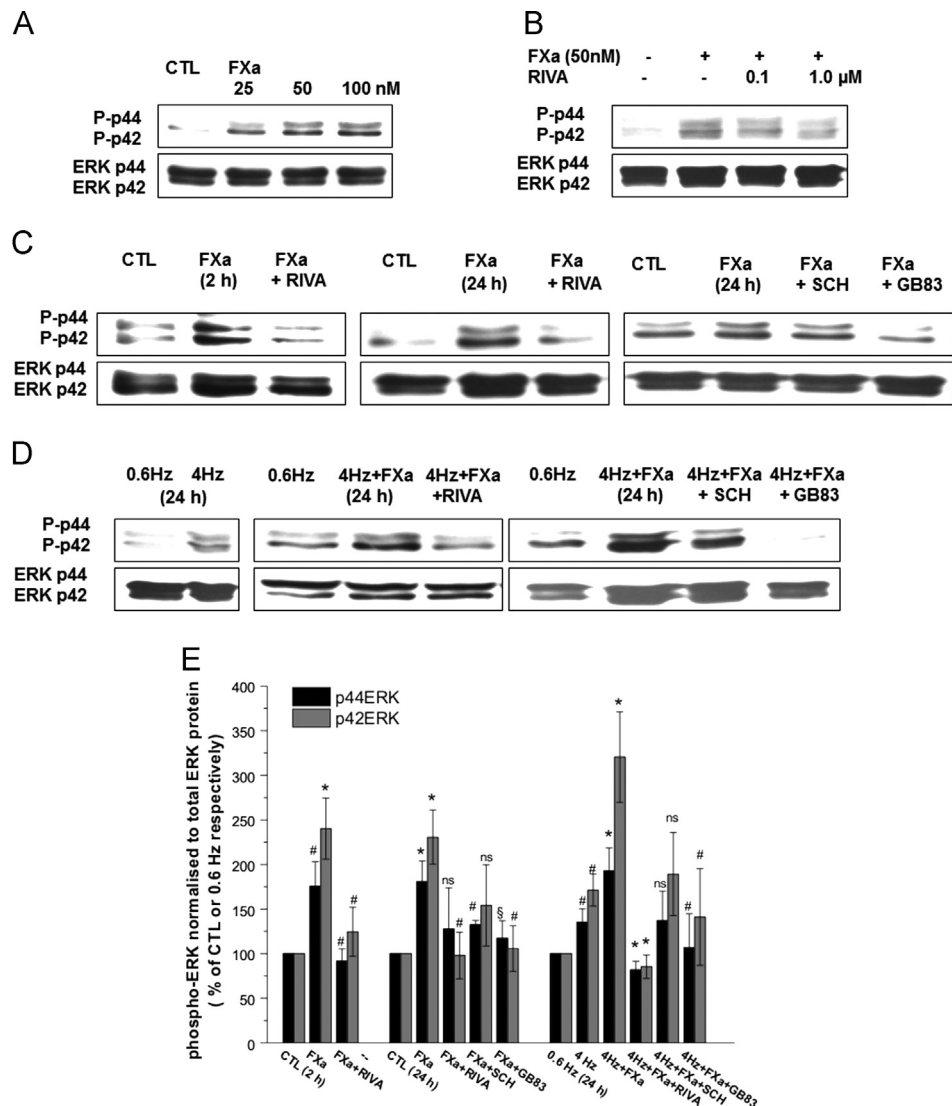
Fig. 2. Activation of ERK in the response to stimulation with FXa and pacing. The phosphorylation state of ERK was analysed using Western blots. (A) FXa activated ERK in a concentration-dependent manner. Human atrial slices were cultivated with different FXa concentrations for 2 h. The immunoblot is representative of two independent experiments. (B) Inhibition of FXa-dependent ERK activation by Rivaroxaban (Riva). Human atrial slices were preincubated with 0.1 and 1 μM Riva for 1 h before the exposure to 50 nM FXa for an additional 2 h. The immunoblot is representative of three independent experiments. (C) Activation of ERK in the response to FXa. Human atrial slices were exposed to 50 nM FXa without or with 1 μM Riva for 2 h ($n=6$ vs. CTL), and to 50 nM FXa for 24 h without ($n=10$) or with 1 μM Riva ($n=7$ vs. CTL), or 10 μM SCH ($n=3$ vs. CTL), or 10 μM GB83 ($n=3$ vs. CTL). (D) Activation of ERK in the response to pacing and FXa. Atrial slices were paced at 4 Hz in the presence of 50 nM FXa ($n=10$ vs. 0.6 Hz) and in the presence of FXa and 1 μM Riva ($n=5$ vs. 4.0 Hz+FXa), or 10 μM SCH ($n=5$ vs. 4.0 Hz+FXa), or 10 μM GB83 ($n=5$ vs. 4.0 Hz+FXa) for 24 h. Further experiments were done to analyse the effect of rapid pacing alone ($n=4$ vs. 0.6 Hz). (E) Quantification of phospho-ERK protein contents by densitometric analysis of Western blots. Results are expressed as means ± S.E.M, compared to unpaced control (CTL), or 0.6 Hz control for pacing experiments respectively. *$P < 0.01$, #$P < 0.05$, and §$P = 0.07$.

4. Discussion

The present study provides evidence that FXa acts as a mediator of inflammatory signalling in human atrial tissue. Moreover, we describe FXa effects in a model of atrial tachyarrhythmia. We can show that application of FXa and atrial tachyarrhythmia act synergistically to increase myocardial expression of protease-activated receptors, followed by activation of MAP kinase pathway (ERK1/2) and NF-κB pathway. As a result, expression of inflammatory molecules: IL-8, ICAM-1, and endothelial factors like prooxidative LOX-1 and prothrombotic PAI-1 are upregulated. We have shown that blockade of FXa by Rivaroxaban abolished FXa- and tachycardia-induced remodelling process in atrial tissue. Furthermore, protease-activated receptors have emerged as powerful targets for preventing molecular changes in atrial tissue.

FXa mediates cellular signalling via protease-activated receptor 1, or protease-activated receptor 2, or both, depending on cell type

and cofactor expression. In fact, studies on dermal fibroblasts and endothelial cells have demonstrated that FXa induced secretion of cytokines via activation of both receptors (Senden et al., 1998; Bachli et al., 2003). However, in lung fibroblasts, protease-activated receptor 1 played a dominant role in FXa-induced responses (Scotton et al., 2009). In contrast, in human vascular smooth muscle cells (SMCs), FXa (50 nM) increased only transcriptional expression of protease-activated receptor 2 (Jobi et al., 2011). Moreover, Jobi et al. (2011) demonstrated that FXa promoted PAR2 mRNA stabilisation through increased human antigen R/PAR2 mRNA binding. The effects of FXa on the atrial tissue have not been investigated previously. Our study revealed that the exposure of resting atrial slices to FXa resulted in an increase in transcriptional expression of protease-activated receptor 2. In contrast, FXa alone had no specific effect on the transcriptional expression of protease-activated receptor 1. However, the regulatory impact of the specific antagonist of protease-activated
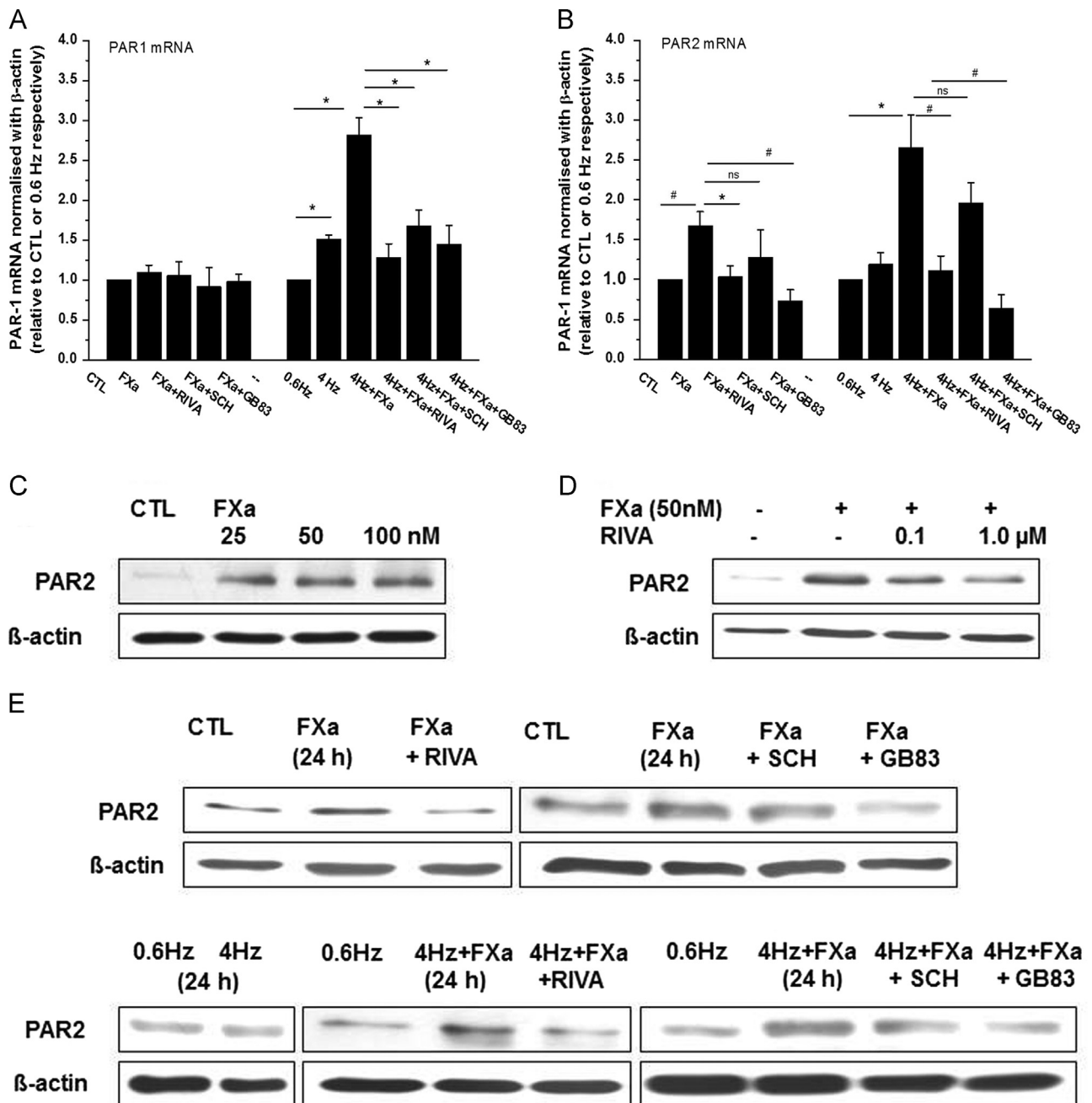
Fig. 3. Expression of protease-activated receptors in response to stimulation with FXa and rapid pacing. The expressions of PAR1 (A) and PAR2 (B) were analysed using RT-PCR. Human atrial slices were exposed to 50 nM FXa for 24 h without ($n=9$) or with 1 μM Riva ($n=6$ vs. CTL), or 10 μM SCH ($n=3$ vs. CTL), or 10 μM GB83 ($n=3$ vs. CTL). Then, atrial slices were stimulated in electrical field at 4 Hz in the presence of 50 nM FXa ($n=7$ vs. 0.6 Hz) and in the presence of FXa and 1 μM Riva ($n=4$ vs. 4 Hz+FXa), or 10 μM SCH ($n=3$ vs. 4.0 Hz+FXa), or 10 μM GB83 ($n=3$ vs. 4.0 Hz+FXa) for 24 h. In addition, further experiments were done to analyse the effects of rapid pacing alone ($n=5$ vs. 0.6 Hz). Results are expressed as means ± S.E.M, compared to unpaced control (CTL), or 0.6 Hz control for pacing experiments respectively. (C) The PAR2 protein expression was analysed using Western blots. FXa increased PAR2 protein content in the response to FXa (C, upper panel). The immunoblot is representative of two independent experiments. Rivaroxaban (Riva) inhibits PAR2 protein level in a concentration-dependent manner. Human atrial slices were preincubated with 0.1 and 1 μM Riva for 1 h before the exposure to 50 nM FXa for an additional 2 h. Increase of PAR2 protein in the response to FXa (C, middle panel). Human atrial slices were exposed to 50 nM FXa for 24 h without or with 1 μM Riva, or 10 μM SCH. (C, lower level) Increase of PAR2 protein in the response to pacing and FXa. Atrial slices were paced at 4 Hz in the presence of 50 nM FXa and in the presence of FXa and 1 μM Riva, or 10 μM SCH, or 10 μM GB83 for 24 h. The immunoblots are representative of three independent experiments. $^*P < 0.01$, and $^{\#}P < 0.05$.

receptor 1 on the PAI-1 and IL-8 expression, strong suggested an involvement of receptor type 1 in the cellular response to FXa. Of note, atrial tachyarrhythmia per se induced upregulation of protease-activated receptor 1 at mRNA level. Interestingly, the combined stimulation with tachyarrhythmia and FXa caused synergistic upregulation of protease-activated receptors at the transcriptional level. This association between tachycardia and

the elevated expression of protease-activated receptor has not been investigated so far.

The ERK–MAPK activation is used as a marker of activation of protease-activated receptors (Ossovskaya and Bunnett, 2004) and is accompanied by a wide range of PAR-mediated cellular processes including fibrosis, cellular proliferation, inflammatory signalling (Borensztajn et al., 2009), and hypertrophy (Pawlinski et al., 2007).
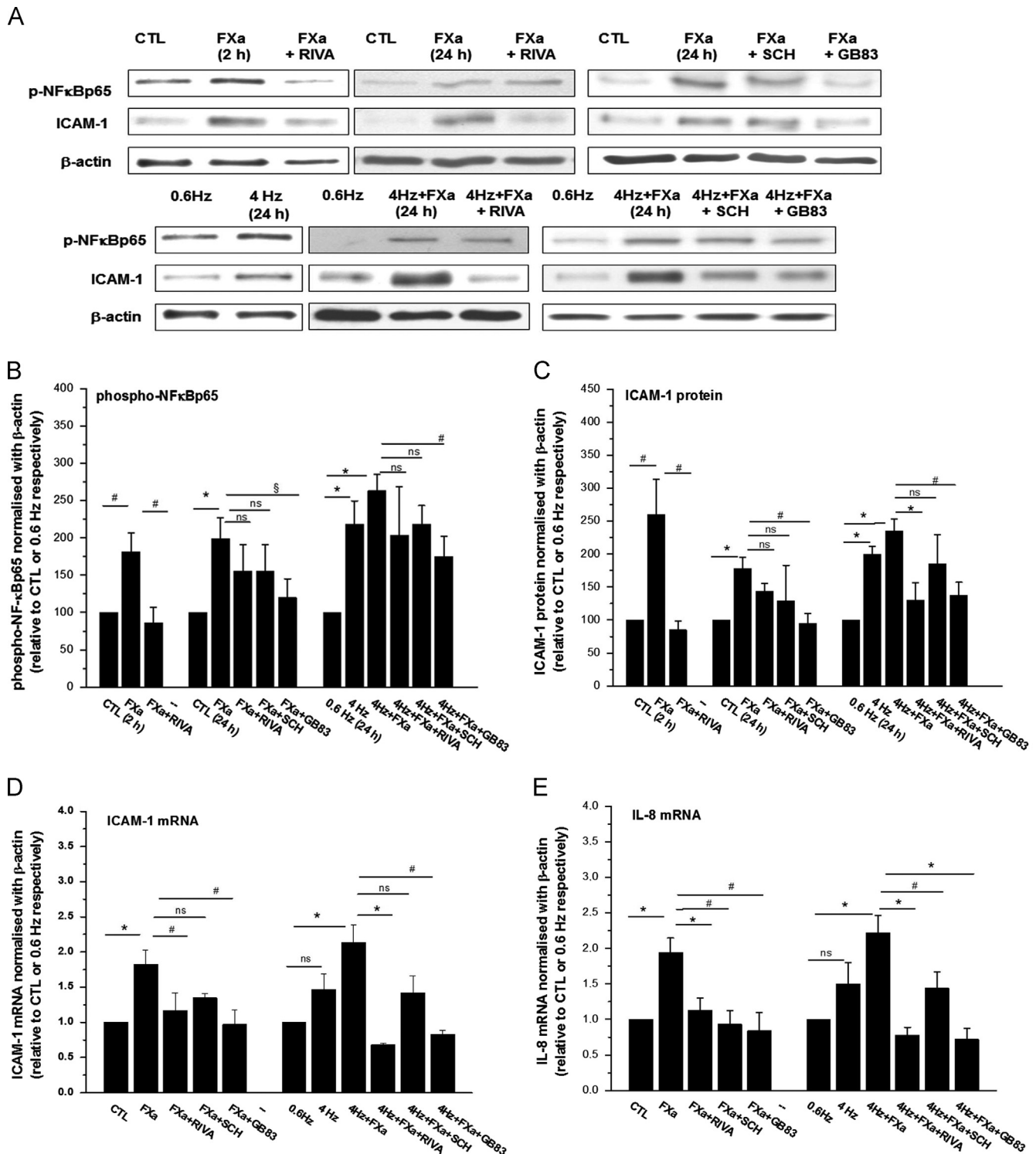
Fig. 4. Activation of NF-κB signalling in the response to stimulation with FXa and rapid pacing. The phosphorylation at Ser-536 of NF-κBp65 and ICAM-1 protein expression were analysed using Western blots. (A, upper panel) Human atrial slices were exposed to 50 nM FXa without or with Riva for 2 h ($n=6$ vs. CTL), and to 50 nM FXa for 24 h without ($n=10$ vs. CTL), and with 1 μM Riva ($n=7$ vs. CTL), or 10 μM SCH ($n=3$ vs. CTL), or 10 μM GB83 ($n=3$ vs. CTL). (A, lower panel) Then, atrial slices were paced at 4 Hz in the presence of 50 nM FXa ($n=10$ vs. 0.6 Hz) and in the presence of FXa and 1 μM Riva ($n=5$ vs. 4.0 Hz+FXa), or 10 μM SCH ($n=5$ vs. 4.0 Hz+FXa), or 10 μM GB83 ($n=5$ vs. 4.0 Hz+FXa) for 24 h. In addition, further experiments were done to analyse the effects of rapid pacing alone ($n=4$ vs. 0.6 Hz). Quantification of phospho-NF-κBp65 (B) and ICAM-1 (C) protein contents was carried out by densitometric analysis of Western blots. (D, E) Additionally the expression of proinflammatory cytokine, IL-8 and adhesion molecule, ICAM-1 were analysed using RT-PCR after exposition to FXa ($n=9$ vs. CTL) and to FXa and pacing ($n=8$) after 24 h. Results are expressed as means ± S.E.M, compared to unpaced control (CTL), or 0.6 Hz control for pacing experiments respectively. $^{*}P<0.01$, $^{#}P<0.05$, $^{§}P=0.07$.

The increasing evidence has shown that FXa elicits the inflammatory response through the activation of the transcription factor NF-κB (Riewald and Ruf, 2001; Jiang et al., 2011; Shimizu et al., 2004; Matsushita et al., 2006). Numerous inflammatory genes are regulated by the NF-κB family of transcription factors including proinflammatory cytokines and adhesion molecules (Hoberg et al., 2004). In human endothelial cells, FXa increases the expression of adhesion molecules (ICAM-1, VCAM-1) and production of proinflammatory
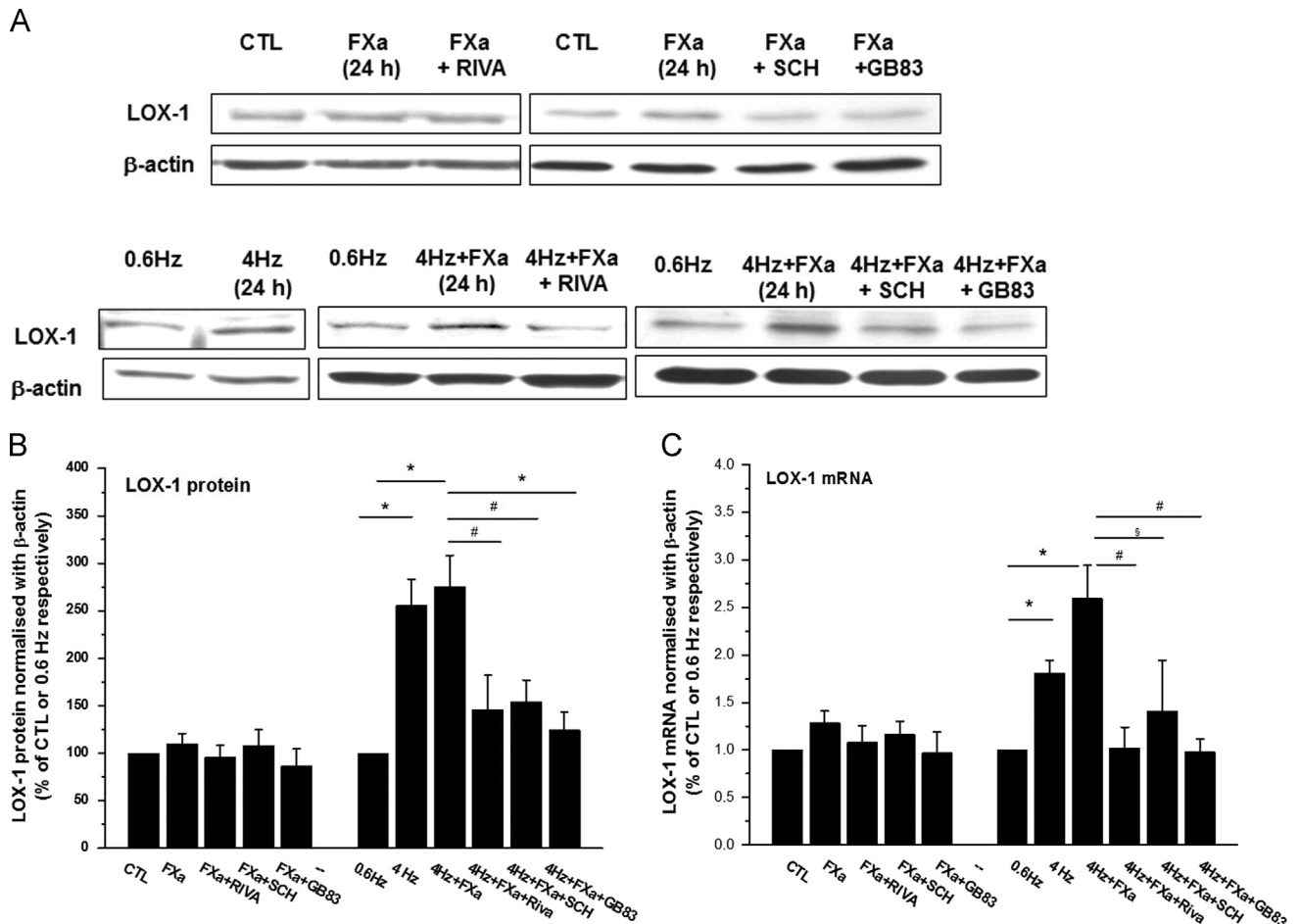
Fig. 5. Induction of LOX-1 in the response to stimulation with FXa and pacing. Human atrial slices were exposed to 50 nM FXa without ($n=10$ vs. CTL) and with 1 μM Riva ($n=7$ vs. CTL), or 10 μM SCH ($n=3$ vs. CTL), or 10 μM GB83 ($n=3$ vs. CTL) for 24 h. Then, atrial slices were stimulated in electrical field at 4 Hz in the presence of 50 nM FXa ($n=10$ vs. 0.6 Hz) and in the presence of FXa and 1 μM Riva ($n=5$ vs. 4.0 Hz+FXa), or 10 μM SCH ($n=5$ vs. 4.0 Hz+FXa), or 10 μM GB83 ($n=5$ vs. 4.0 Hz+FXa) for 24 h. Furthermore, effects of rapid pacing were studied ($n=4$, 4 Hz vs. 0.6 Hz). (A) LOX-1 expression was analysed using Western blots. (B) Quantification of LOX-1 protein contents by densitometric analysis of Western blots. (C) Quantitative analysis of LOX-1 mRNA expression using RT-PCR. Results are expressed as means ± S.E.M, compared to unpaced control (CTL), or 0.6 Hz control for pacing experiments respectively. *$P < 0.01$, and #$P < 0.05$.

cytokines (IL-6, IL-8, MCP-1) (Senden et al., 1998). In human SMCs, FXa causes a dose-dependent induction of IL-6 and tissue factor expression (McLean et al., 2001). In mouse fibroblast cell line, FXa-induced ERK1/2 phosphorylation was accompanied by the secretion of the cytokines, and differentiation into myofibroblasts (Borensztajn et al., 2009). In our study, we have shown that FXa induced the inflammatory response by activation of ERK1/2 and NF-κB and this stimulation in atrial tissue resulted in sustained activation (phosphorylation) throughout a 24 h period. This result contrasts with other studies showing a transient character of phosphorylation events upon exposure to FXa. In our work, we have used tissue slices composed predominantly of atrial cardiomyocytes and fibroblasts that may interplay during the exposure to FXa. A study by Rauch et al. (2004) reported that FXa released a basic fibroblast growth factor (bFGF) into pericellular matrix (Rauch et al., 2004). The autocrine transactivation of FGF receptor-1 resulted in sustained ERK phosphorylation in vascular SMCs (Rauch et al., 2004; Pratsinis and Kletsas, 2007), which might implicate the bimodal effect of FXa, in general. In line with the activation of MAPK and NF-κB signalling in response to FXa induction, we observed an increase of proinflammatory molecule like ICAM-1 and IL-8, and prothrombotic PAI-1 in atrial tissue. Of importance, our results suggest that application of FXa and atrial tachyarrhythmia acted synergistically to induce inflammatory and prothrombotic tissue effects. One key element of protease-activated receptors signalling depends on modulation of calcium

homoeostasis. In support of this hypothesis, a sustained increased $[Ca^{2+}]_i$, which depends on extracellular Ca^{2+} influx, was described in a subpopulation of the platelets after stimulation with thrombin plus collagen and was associated with generating procoagulant platelet surfaces (Keuren et al., 2005). However, further research is needed to fully elucidate all mechanisms responsible for regulation of protease-activated receptor signalling.

Naturally and synthetic occurring FXa inhibitors exert strong anticoagulant activity and have been shown to be potent antithrombotic agents in animal models of venous and arterial thrombosis (Wong et al., 1996; Perzborn et al., 2007). Independent of the antithrombotic actions, several studies demonstrated that FXa inhibitors exert anti-inflammatory effects (McLean et al., 2001; Hoberg et al., 2004). DX-9065a, the first generation of FXa inhibitors, prevented FXa-induced IL-6 mRNA expression in human gingival fibroblasts and was considered as a useful drug for periodontal disease. Recently Zhou et al. (2011) have demonstrated that long-term administration of Rivaroxaban to apolipoprotein E-deficient mice reduced the expression of inflammatory mediators in aortic tissue (Zhou et al., 2011).

Cardiac tachyarrhythmia is known to induce significant electrophysiological and structural changes in atrial tissue, which themselves may contribute to the persistence and aggravation of atrial fibrillation (Schotten et al., 2011; Goette et al., 1996). Alterations in the intracellular calcium and activation of oxidative
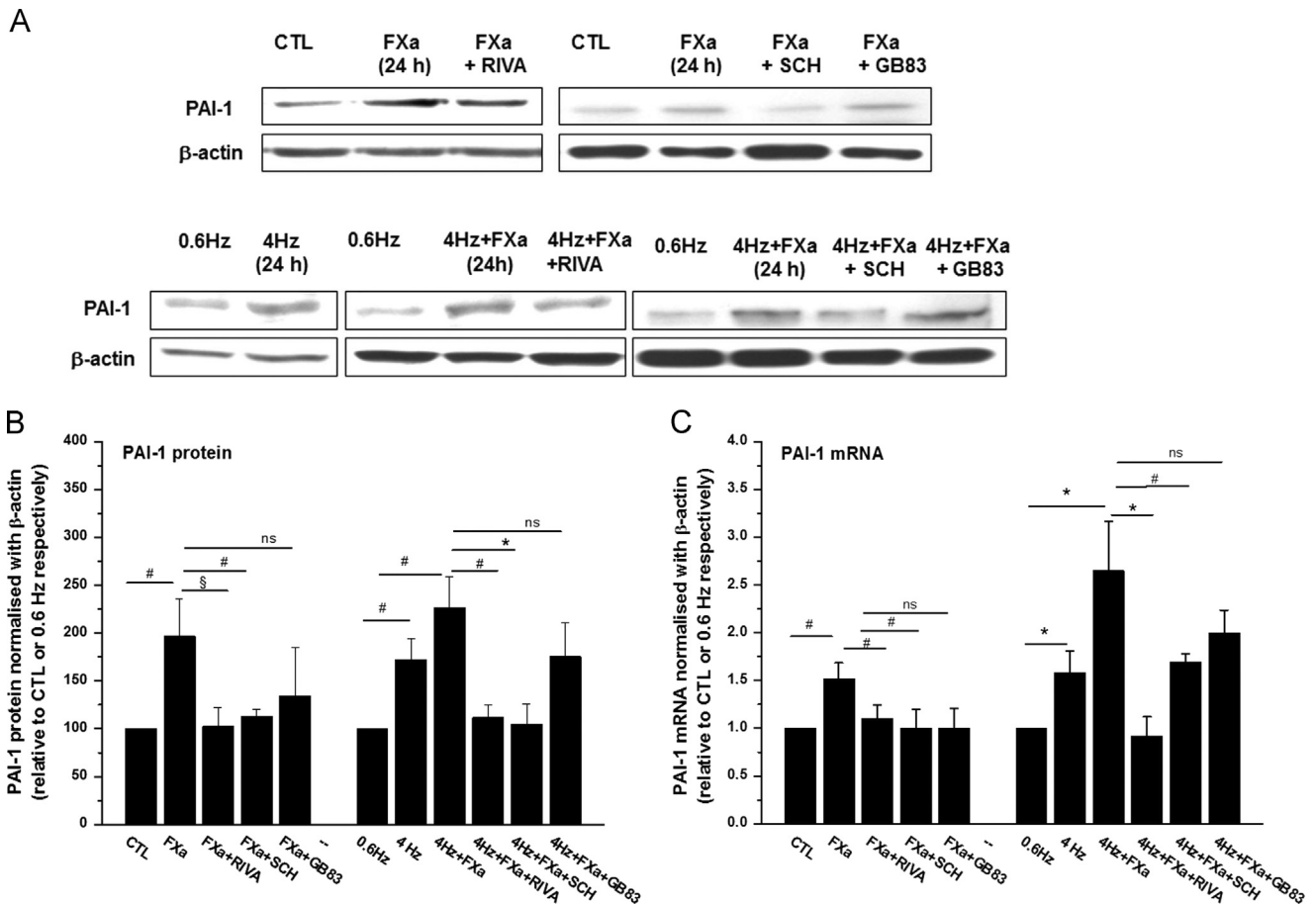
Fig. 6. Effects of the stimulation with FXa and pacing on the expression of PAI-1. (A) PAI-1 protein expression was analysed using Western blots. Human atrial slices were exposed to 50 nM FXa without ($n=10$ vs. CTL) and with 1 μM Riva ($n=7$ vs. CTL), or 10 μM SCH ($n=3$ vs. CTL), or 10 μM GB83 ($n=3$ vs. CTL).for 24 h (A, upper panel). Then, atrial slices were stimulated in electrical field with the frequency of 4 Hz in the presence of 50 nM FXa ($n=10$ vs. 0.6 Hz) and in the presence of FXa and 1 μM Riva ($n=5$ vs. 4.0 Hz + FXa), or 10 μM SCH ($n=5$ vs. 4.0 Hz + FXa), or 10 μM GB83 ($n=5$ vs. 4.0 Hz + FXa) for 24 h. Furthermore, effects of rapid pacing were analysed ($n=4$, 4 Hz vs. 0.6 Hz). (B) Quantification of PAI-1 protein contents was carried out by densitometric analysis. Additionally analysis using RT-PCR were performed to determine the expression of prothrombotic PAI-1 (C). Results are expressed as means $\pm$ S.E.M, compared to unpaced control (CTL), or 0.6 Hz control for pacing experiments respectively. $^{*}P < 0.01$, and $^{\#}P < 0.05$, $^{\S}P=0.07$.

stress signalling have been identified to trigger atrial remodelling during atrial fibrillation (Bukowska et al., 2008; Dudley et al., 2005, Schild et al., 2006). Recently, we were able to show the impact of the nuclear factor kappa B (NF-κB) pathway in the process of rapid pacing induced oxidative stress (Bukowska et al., 2008). In line with the activation of NF-κB signalling, we observed an increase in expression of adhesion molecules like ICAM-1, but also LOX-1 in atrial tissue from patients with atrial fibrillation and in in vitro paced atrial tissue slices. These findings strongly imply that oxidative signalling accounts for endocardial activation followed by an increased risk of atrial thrombus formation during tachyarrhythmia. Endothelial damage and prothrombotic endothelial alteration are present in fibrillating atria (Schotten et al., 2011). They are a prerequisite for the development of atrial clots, since atrial thrombi always start to grow from the atrial wall.

5. Study limitations

The present study has several limitations. First off all, the human tissue slices were prepared from biopsies obtained only from right appendages. Although pathological alterations might be more pronounced in left atria, there is no data supporting the principally different employment of molecular signal cascades within left and right atria. Second, all patients had coronary artery or valve disease with concomitant hypertension and/or diabetes

mellitus. Thus, in contrast to animal studies, no healthy controls were used. Third, our study was limited by inability to perform measurements of intracellular calcium level in tissue slices during culturing or stimulation due to technical problems involving the thickness of the slices and dimension of devices used in our "in vitro" model. Finally, the high concentration of FXa administrated in "in vitro" experiments might rouse reasonable scepticism of the "in vivo" relevance. In human plasma, the concentration of zymogen factor X is 140 nM. Thus, concentration of activated factor X could theoretically reach high concentration at the sites of local FXa activation and influence the intracellular signalling in vivo. The gene induction required 10 nM of FXa, a concentration consistent with cell membrane-binding affinity for FXa (Riewald and Ruf, 2001). Obviously, future experiments should elucidate the in vivo relevance of FXa in atrial arrhythmia.

6. Conclusions

The present study provides evidence that in human atrial tissue, FXa acts a mediator of inflammatory signalling via activation of protease-activated receptors 1 and 2. Most importantly, the synergistic action of FXa and atrial tachyarrhythmia results in a potentiated response involving the increase of inflammatory and oxidative stress molecules, which create an inflammatory, prothrombotic status in atrial tissue. Importantly, the FXa

induced inflammatory signalling can be substantially attenuated by FXa antagonist, Rivaroxaban. Thus, FXa and protease-activated receptors appear as novel therapeutic targets to prevent atrial remodelling.

Disclosures

Acknowledgements

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