Supplementary materials

Methods and materials

Establishing the atrial fibrillation (AF) model by rapid atrial pacing (RAP)

The AF model was prepared partly as described previously (1, 2, 3, 4) with some modifications. Briefly, rats were anesthetized by the intra-peritoneal injection of pentobarbital sodium (50 mg/kg), and body surface II-lead ECG was continuously monitored using a polygraph system (Power Lab 4/35, AD Instruments Shanghai Trading Co, Shanghai, China) throughout the study. A supplementary injection (0.1 ml/100 g) of pentobarbital sodium solution (3%) was given every 60–90 min to maintain the state of anaesthesia. After a 10-min equilibration period, a clinically used 5F pacing catheter with two 1-mm ring electrodes and an inter-electrode distance of 5mm was inserted orally into the oesophagus of the rats approximately 7 cm and positioned to ensure constant atrial capture under the guidance of B-mode ultrasound (Siemens Medical Systems, Mountain View, CA, USA). When the electrode captured the middle part of the left atria, the pacing current was adjusted to rectangle in shape, 60v (about 1.5-times the diastolic threshold voltage) and 6-ms width. The frequency was set at 30Hz sustained for 1 h, 3 h or 8 h using an electrical stimulator and an isolator (JL-C2, Jia Long Instrument Factory, Shanghai, China).

Preparation of platelets (PLTs) and immunofluorescence

Platelets were isolated as previously described (5, 6). After rats anesthetized, blood was collected by cardiac puncture. Blood was drawn into syringes containing 1/10th of the final blood volume of sodium citrate anticoagulant (Sigma-Aldrich, St. Louis, MO, USA). The anti-coagulated blood was centrifuged at 200×g for 10 min to obtain platelet-rich plasma (PRP). Platelets were purified from PRP by centrifugation at 1000×g in the presence of prostacyclin (0.1 μg/ml) and washed twice with 10 ml of modified HEPES/Tyrode buffer (129 mM NaCl, 0.34 mM Na2HPO4, 2.9 mM KCl, 12 mM NaHCO3, 20 mM HEPES, 5mM glucose, 1 mM MgCl2; pH 7.3) containing 0.1μg/ml prostacyclin. To selectively pellet erythrocytes, platelets were resuspended in the same buffer and centrifuged twice at 120×g for 3 min. Erythrocytes content was 1% as counted using a hemocytometer. The purified platelets were resuspended in the medium described above containing prostacyclin (0.1 μg/ml) and incubated at 37°C and 5% CO2. To measure the effect of thrombin on P-selectin expression, platelets were washed and incubated in serum-free DMEM for 6 h and then stimulated with various concentrations of thrombin (T5772, Sigma-Aldrich, St. Louis,
MO, USA) for 10 mins, and P-selectin expression on platelets was measured by immunofluorescence labelling. After stimulation with thrombin, PLTs were fixed in 4% paraformaldehyde for 20 min at 4°C on confocal culture dishes. Then, fixed PLTs were washed three times with phosphate-buffered saline (PBS) for 5 min each. After incubation with 1% bovine serum for 20 min, the PLTs were incubated with rabbit anti-rat P-selectin antibodies (LS-C393361, Life Span, Seattle, WA, USA) overnight at 4°C. Next, cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (A0423, Beyotime, Shanghai, China) for 30 min in a dark environment. Images were visualised on a confocal microscope within a week. The fluorescence intensity of P-selectin was quantified using Image-Pro Plus 6.0 and the data were expressed as a percentage of fluorescence intensity.

**Cell culture and immunofluorescence**

A human umbilical vein endothelial cell (HUVEC) line, (Geneseed, Guangzhou, China) was maintained in Dulbecco’s modified Eagle’s Medium (DMEM, Gibco/Life Technologies, Carlsbad, CA, USA) containing 10% foetal calf serum and a mixture of glutamine, penicillin and streptomycin at 37°C in a 5% CO₂ humidified incubator. After stimulation with LPS or P-selectin, cells were fixed in 4% paraformaldehyde for 20 min at 4°C on confocal culture dishes. Then, fixed cells were washed three times with PBS for 5 min each. After incubation with 1% bovine serum for 20 min, the cells were incubated with rabbit anti-human P-selectin and anti-human TF antibodies (ab178424 and ab151748; both from Abcam, Cambridge, MA, USA) overnight at 4°C. Next, cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (A0423, Beyotime, Shanghai, China) for 30 min in a dark environment. Then 4’, 6-diamidino-2-phenylindole (DAPI) (C1005, Beyotime, Shanghai, China) was added to the cells to stain the nuclei for 15 min in a dark environment. Images were visualised on a confocal microscope within a week. The fluorescence intensity of P-selectin and TF was quantified using Image-Pro Plus 6.0 and the data were expressed as a percentage of fluorescence intensity.

**Results**

**Immunohistochemical (IHC) staining of the left atrial appendage (LAA) tissue**

The staining intensity of P-selectin, CD41, fibrinopeptide A (FPA) and tissue factor (TF) of LAA tissue was higher in the RAP 8 h group than the Sham group (P<0.05; Suppl. Fig. 1).
References


Suppl. Table 1.

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*P<0.05

Suppl. Table 1. No changes in systolic blood pressure in the process of rapid atrial pacing (RAP), heart rate (HR) accelerated after successful generation of atrial fibrillation model.
Suppl. Figure 1.
Suppl. Figure 2.

Suppl. Figure 3.

Figure legends

Suppl. Figure 1. Histopathologic examination of the left atrial appendage (LAA). (A) Representative images of the LAA, as visualised by hematoxylin and eosin (H&E) or after
labelling with antibodies against P-selectin, CD41, fibrinopeptide (FPA) and tissue factor (TF). (B) Quantitative analysis of P-selectin (a), CD41 (b), FPA (c) and TF (d) staining (n=6 per group). *P<0.05 vs. the Sham group. Statistical analyses were performed using independent-samples t-tests between two groups.

**Suppl. Figure 2. Representative SEM (scanning electron microscope) images of LAA (left atrial appendage) endocardial surface.** A) Morphology of endocardial surface and deposition of platelets (PLTs), red blood cells (RBCs) and fibrin on the endocardium were observed using SEM in Sham, rapid atrial pacing (RAP) 1 h RAP 3 h and RAP 8 h groups. (PLT: yellow arrow; RBC: red arrow; fibrin: white arrow) B) Quantitative analysis of PLT and RBC on the LAA endocardium. (means ± SD, n=3 per group, *P<0.05) C) Representative images of thrombus of left atrial (LAA) in RAP 8 h group rats stained with haematoxylin and eosin (H&E). Statistical analyses were performed using independent-samples t-tests between two groups and one-way ANOVA analysis followed by Bonferroni’s post-hoc tests (Dunnett’s T3 tests were used without equal variance) between multiple groups.

**Suppl. Figure 3. Representative image of P-selectin immunohistochemical (IHC) detection of P-selectin inhibited group (PSI) group.**