Thrombomodulin and Tissue Factor Pathway Inhibitor in Endocardium of Rapidly Paced Rat Atria

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Background—Atrial fibrillation (AF) is well known as one of the cardiogenic causes for thromboembolism. Although decreased flow and hypercoagulable state of the blood in the fibrillating atrium have been emphasized as the underlying mechanisms, endocardial dysfunction in maintaining the local coagulation balance could also contribute to the thrombogenesis in AF.

Methods and Results—The paroxysmal AF model was created by rapid atrial pacing in anesthetized rats. To test the hypothesis that AF induces local coagulation imbalance by disturbing the atrial endocardial function, the gene expression of intrinsic anticoagulant factors, thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI), were determined by means of ribonuclease protection assay, Western blotting, and immunohistochemistry. Rapid atrial pacing for 8 hours significantly decreased TM and TFPI mRNA levels in the left atrium but not in the ventricle, leading to the downregulation of their immunoreactive proteins. Immunohistochemical analysis revealed that TM and TFPI were expressed predominantly in the endocardial cells of the normal atrium, presumably preventing local blood coagulation, and that rapid atrial pacing induced the loss of TM and TFPI expression in the endocardium, leading to deficiency in anticoagulant barriers between the atria and the blood.

Conclusions—Rapid atrial pacing acutely downregulated the gene expression of TM and TFPI in the atrial endocardium, thereby inducing local coagulation imbalance on the internal surface of the atrial cavity. These results would support the validity of supplement of anticoagulant molecules deficient in AF. (Circulation. 2003;108:2450-2452.)

Key Words: fibrillation ■ anticoagulants ■ endocardium

With the increasing number of aged people, atrial fibrillation (AF) has gained more attention as an important cardiogenic cause for thromboembolism.1 During AF, it is feasible for thrombus formation to occur in the left atrial appendage, resulting in the increase in the incidence of stroke in patients with AF.2 Decreased blood flow with hypercoagulable state in the left atrium has been attributed to the likelihood of clotting formation in the fibrillating atria, as demonstrated in many previous studies.3,4 In vascular thrombi, however, endothelial dysfunction is also known to play a significant role in addition to the decreased blood flow and hypercoagulability, as shown in the well-known Virchow’s triad. Likewise, we could hypothesize that atrial endocardial cells could prevent local thrombus formation under normal conditions and that AF might produce the endocardial dysfunction contributing to the local thrombogenesis, besides the deterioration in the blood flow and coagulability. In the present study, to test this hypothesis, we examined the gene expression of intrinsic anticoagulant factors, thrombomodulin (TM) and tissue factor pathway inhibitor (TFPI), in the endocardium of rapidly paced atria.

Methods

Preparation of Atrial Tachycardia Models

Sprague-Dawley rats (Charles River Japan, Inc, Yokohama, Japan), 12 weeks of age, were used in the present study. Atrial tachycardia rat model was prepared as shown in our previous study.5 Briefly, a quadripolar electrode catheter (1.5F) was introduced through the cervical vein for pacing and recording in anesthetized rats. Stimulation at a frequency of 1200 bpm was performed with 2-ms rectangular pulses, with the use of a programmable stimulator and a constant current source (SEN 7203 and SS401J, Nihon Kohden). Sham-operated animals underwent an identical procedure without stimulation. Hearts were removed after 8-hour pacing, the left atrial appendages were removed within 30 seconds, and material for RNA and protein analysis was immediately frozen in liquid nitrogen.

Preparation of DNA Templates

DNA templates of TM, tissue factor (TF), and TFPI were prepared by reverse transcriptase–polymerase chain reaction (Access RT-PCR system, Promega) from total RNA isolated from the rat atrium. The amplified cDNA fragments were subcloned into pCR II vectors (Invitrogen) and confirmed by sequencing. The primers specific for each gene, based on the known sequence, were forward primer 5’-GACGCTGCAAAACTTCTGAGGGAT-3’ and reverse primer 5’-TCCTCggggttcaagtctccctcTA-3’ for TM; forward primer 5’-GATTG-TGTATTTCTACACGCGGGAA-3’ and inverse

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primer 5′-CACAAGGATGCTCCAAG-CAGAAAT-3′ for TF; forward primer 5′-CAATGGGACTATGATGGCCCTTCA-3′ and inverse primer 5′-AATCATAGTGAACACCTTATAG-3′ for TFPI. These plasmids were used to synthesize the antisense digoxigenin-labeled RNA probes.

RNA Preparation and RNase Protection Assay
The levels of the mRNAs were assayed with the use of the RNase protection assay with RPA III kit (Ambion), as described previously.5 Chemiluminescent signals were quantified with the use of the lumino-image analyzer (ATTO LightCapture AE-6960, ATTO). The cyclophilin signals were used as internal control.

Western Blot Analysis and Immunohistochemistry
Total proteins (30 μg) extracted from the left atrial appendages were fractionated on SDS-PAGE gels and transferred to PVDF membranes (Boehringer Mannheim). The membrane was incubated with polyclonal anti-TM (American Diagnostica Inc) and anti-TFPI (The Chemo-Sero-Therapeutic Research Institute) antibody obtained from rabbit and subsequently with goat anti-rabbit immunoglobulin G conjugated to alkaline-phosphatase (Boehringer Mannheim). The immunohistochemistry was performed in cryostat sections (8 μm thick) by Dako EnVision+ Systems (Dako). Polyclonal anti-CD31 antibody from mouse (BD Biosciences Pharmingen) was used as a marker of endocardial cells.

Statistical Analysis
The mean values of the mRNA and protein levels were compared by means of the unpaired t test. Statistical significance was set at a probability value <0.05.

Results
Downregulation of TM and TFPI Gene Expression in Rapidly Paced Atria
Figure 1A shows the mRNA expression of TM, TF, and TFPI in the left atrium and ventricle. The mRNA levels of these genes were not affected by rapid pacing in the ventricle. However, those of TM and TFPI were both decreased significantly in the rapidly paced left atrium (P = 0.033 and 0.043, respectively, n=10), although TF mRNA was not altered. The decreases in the mRNA level were remarkable for TFPI (≈65%) and relatively small for TM (≈35%). Western blot analysis revealed that both TM and TFPI immunoreactive proteins were also decreased significantly by rapid pacing (Figure 1B) by ≈30% for TM and ≈40% for TFPI (P = 0.037 and 0.008, respectively, n=10).

Localization of Downregulated TM and TFPI
Immunohistochemical analysis revealed the distribution of TM and TFPI in the left atrium. In sham-operated animals, both TM and TFPI proteins were detected predominantly in the endocardial CD31-positive cells (Figure 2, A through C), relative to the adjacent atrial myocytes. In rapidly paced hearts, however, TM and TFPI expression was much attenuated in the CD31-positive cells in the endocardium, thus leading to the apparent loss of TM and TFPI in the internal surface of the left atrial cavity (Figure 2, D through F).

Discussion
The major finding of the present study was that rapid atrial pacing acutely downregulated the gene expression of intrinsic anticoagulant factors, TM and TFPI, mainly in the atrial endocardium. Thus, AF could induce acute deficiency in the endocardial function protective for clotting, which could be partly associated with local thrombogenesis.

Feasibility of thrombus formation in the fibrillating atria is a well-known phenomenon explaining the increased
incidence of stroke in AF patients. Decreased laminar flow and hypercoagulable state of the blood have been emphasized as important involved mechanisms. However, a recent study,6 for the first time, has reported the possible involvement of the endocardial dysfunction: AF decreased the nitric oxide synthase expression in the atrial endocardium. Our present results would also support this concept of endocardial dysfunction during AF. The atrial endocardium should act as an anticoagulant barrier during sinus rhythm, as shown by the immunohistochemical analysis that the surface of the endocardium was almost fully covered with anticoagulant molecules, TM and TFPI. However, rapid atrial excitation for several hours induced the decrease in their gene expression, apparently stripping these important anticoagulant barriers.

Both TM and TFPI are known to play a pivotal role in maintaining the normal coagulation balance.7,8 They are expressed mainly in the surface of the endothelial cells of the vasculature and prevent the blood clotting on the internal surface of the vessels. Endothelial TM forms a complex with thrombin and thereby changes its substrate specificity as an intrinsic thrombin inhibitor. Thrombin bound to TM cannot convert fibrinogen to fibrin and moreover activate protein C, a major anticoagulant protein.7 TFPI is a direct inhibitor of factor Xa activity and is a factor X–dependent inhibitor of TF and factor VII/VIIa, thus being the most important physiological inhibitor of TF-dependent coagulation.8 Therefore, both TM and TFPI act as intrinsic anticoagulant barriers between the blood and the vasculature cells. The present results could extrapolate this concept also to the atrium. In normal hearts, TM and TFPI were expressed predominantly in the endocardial surface of the atrium, presumably preventing the thrombus formation on the atrial endocardium. Downregulation of these molecules during AF would promote thrombogenesis by inducing the local coagulation imbalance, in addition to the hypercoagulable state of the blood. Thus, the present data would strengthen the concept of endocardial remodeling by AF, in view of its deficiency in the intrinsic anticoagulant system provided in the heart.

The downregulation of TM and TFPI probably would be multifactorial but resulted from surroundings specific to the atrium because no alterations were observed in the ventricle. Their amounts on the endocardium should depend on the transcription, the rate of synthesis, internalization, and release from the endocardial cell surface. Among these, internalization, known to be constitutive in many other pathological states for TM,8 would be unlikely from our immunohistochemical analysis. Rather, one of the most plausible mechanisms would be downregulation at their gene levels, as shown in the mRNA analysis. Actually, this is consistent with the previous report10 that increased pressure to the vein acutely decreased TM expression level in the venous endothelium, because a slight rise in the atrial pressure was also noted in our rat model.1 However, both TM and TFPI have been reported to be released from the endothelium as a soluble particle,7,8 and the increase in the endocardial release might be also involved. Circulating TM level has been reported to be increased in patients with persistent AF,11 which could be attributed partly to the increased release from the endocardium of the fibrillating atrium.

There has been ample clinical evidence that anticoagulation therapy is more effective for the prevention of thromboembolism in patients with AF than antiplatelet therapy, irrespective of AF types (paroxysmal, persistent, and permanent).12 The present results would strengthen this idea by demonstrating the endocardial coagulation imbalance induced by several-hour atrial tachycardia. When the inherent anticoagulant factors TM and TFPI are lost on the internal surface of the atrium, supplementation of these anticoagulant molecules or their substitutes, including thrombin inhibitors, would be promising.13 These considerations will be assessed by the ongoing large clinical trials using a thrombin inhibitor (Stroke Prevention Using the Oral Direct Thrombin Inhibitor Ximelagatran in Patients With Nonvalvular Atrial Fibrillation [SPORTIF III and V]).14 Moreover, the present results would open a new target for potential therapeutic intervention: restoring the local anticoagulation endothelial milieu. Identification of detailed signaling pathways involved in the downregulation of these molecules—that is, their transcriptional regulatory mechanisms—could produce another new therapeutic strategy of prevention and reversal of the “procoagulation” remodeling of the fibrillating atria.

References
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