EXPERIMENTAL STUDY

Effects of Aging and Hypertension on the Antithrombotic Function of Atrial Endocardium in Rats
Additive Effects to Atrial Fibrillation

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Summary
We have previously reported that atrial endocardial remodeling is induced by atrial fibrillation (AF), and the endocardial dysfunction may be partly responsible for the thrombus formation in the left atrium associated with AF. However, the relationship between the endocardial dysfunction and the epidemiologically determined risk factors of AF-related strokes, including aging, hypertension, and diabetes mellitus, is yet to be elucidated.

To test the hypothesis that aging, hypertension, and diabetes mellitus individually impair the atrial endocardial function in conjunction with AF, we have analyzed the expression of the tissue factor pathway inhibitor (TFPI) and thrombomodulin (TM) in the atrial endocardium in 30-week-old Wister-Kyoto (WKY), 60-week-old WKY, 30-week-old spontaneously hypertensive rats (SHR), and 30-week Goto-Kakizaki (GK) rats during normal sinus rhythm and after rapid atrial pacing at 1200 bpm for 8 hours, using Western blotting and immunohistochemical analysis. Even during sinus rhythm, the TFPI and TM expressions were noted to be remarkably downregulated in the atrial endocardium among 60-week-old WKY rats. In contrast, in SHR rats, only the TFPI expression has significantly decreased, while TM was preserved to the same level of control 30-week-old WKY rats. Rapid atrial pacing significantly reduced the TM and TFPI expression similarly in each model, thereby augmenting the endocardial dysfunction during normal sinus rhythm individually induced by the risk factors themselves prior to AF.

Aging and hypertension, both of which are epidemiologically well-known risk factors for strokes in AF, have been associated with a specific atrial endocardial impairment prior to AF that could additionally disturb the antithrombotic function of the atrial endocardium.

Key words: Atrial tachycardia, Anticoagulants, Endocardial remodeling, Thromboembolism

Atrial fibrillation (AF) has been known as one of the important causes of ischemic strokes and systemic thromboembolisms.1,2) The CHADS 2 and CHA2DS2-VASc scores have been widely used for the risk assessment of stroke in patients with AF.3,4) There have been no supportive data to explain the mechanisms of left atrial (LA) thrombus formation associated with such risk factors. Multiple factors such as blood stasis, endothelial dysfunction, and hypercoagulability as shown in the Virchow’s triad were involved in the clotting formation.5,6) Similar mechanisms might be involved in the thrombus formation associated with AF.5,6) In addition, we previously reported that rapid atrial pacing in an AF experimental rat model acutely reduced the anti-coagulation proteins, tissue factor pathway inhibitor (TFPI), and thrombomodulin (TM)7,8) expressed in the LA endocardium, suggesting endocardial dysfunction might be responsible for the thrombus formation in the left atrium of patients with AF. However, thromboembolic events are rare in the patients without risk factors (i.e., CHA2DS2-VASc score zero) who are usually not indicated for anti-coagulation therapy.4) Therefore, in addition to AF per se, it is speculated that risk factors including aging, hypertension, and diabetes mellitus (DM) synergically contribute to thrombus formation. However, the pathophysiology of thrombus formation associated with AF remains to be determined. Thus, in this study, we aim to investigate whether the risk factors such as aging, hypertension, and DM individually impair the atrial endocardial function in conjunction with AF by using various pathophysiological rat models.

Methods
Preparation of the atrial tachycardia models: Wister-Kyoto (WKY) rats aged 30 weeks (WKY-30W), spontaneously hypertensive (SHR) rats aged 30 weeks (SHR-30 W), WKY rats aged 60 weeks (WKY-60W), and GK rats aged 30 weeks (GK-30W) were used in this present study.

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All of the rats were male. An atrial tachycardia rat model was prepared as shown in our previous study.10-12 Briefly, the rats were anesthetized with pentobarbital (50 mg/kg) and ventilated with a volume-cycled respirator. A quadripolar electrode catheter (1.5 Fr) was introduced through the right cervical vein in anesthetized rats for pacing and recording. Right atrial burst pacing at a frequency of 1,200 bpm was then performed with 2 millisecond rectangular pulses using a programmable stimulator and a constant current source (SEN 7203 and SS401J, Nihon Kohden). Sham-operated animals underwent an identical procedure without right atrial stimulation. The hearts were rapidly removed after 8 hours of pacing, the LA appendages were obtained within 30 seconds, and the material for protein analysis was immediately frozen in liquid nitrogen.

**Measurement of hemodynamic parameters:** The systolic and diastolic pressure and heart rate were determined using a tail-cuff method with a manometer (BP-98A, Softron, Tokyo). To obtain the data, the measurement was conducted at an average of three times.

**Western blot analysis and immunohistochemistry:** The total proteins (30 μg) extracted from the LA 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.), anti-TFPI (the Chemo-Sero-Therapeutic Research Institute), anti-eNOS, and anti-PAI-1 antibodies obtained from rabbits and subsequently with goat anti-rabbit immunoglobulin G conjugated to alkaline-phosphatase (Boehringer Mannheim). The immunohistochemistry was performed in cryostat sections (8 μm in thickness) via the Dako EnVision+ Systems (Dako). Polyclonal anti-CD31 antibody from mice (BD Biosciences Pharmingen) was used as a marker of the endocardial cells in the immunohistochemical analysis. The polyclonal anti-CD34 antibody from mice (BD Biosciences Pharmingen) was used as a marker of the endocardial cells in Western blot analysis.

All animal experiments and maintenance were carried out in accordance to the ethical guidelines suggested by the Institutional Animal Ethics Committee. Data are expressed as the mean ± SD. The mean values between the two groups were compared using unpaired t-tests. The mean value among the groups was compared using an analysis of variance with a Kruskal-Wallis test using Prism 8.0 software (GraphPad software, La Jolla, CA, USA). A two-tailed P-value < 0.05 was considered statistically significant.

### Results

**Hemodynamic parameters in each rat model:** SHR-30 W rat was found to be significantly hypertensive and has increased ratio between heart weight and body weight, indicating ventricular hypertrophy as compared with WKY-30W rat. On the other hand, heart weight of WKY-60W rat was noted to be significantly heavier than that of WKY-30W. However, no significant differences were noted in the ratio between heart weight and body weight between WKY-30W and WKY-60W rats. GK-30W rat was determined to be free from hypertension (Table).

**Expression of TM and TFPI in the atrial endocardium during sinus rhythm:** CD31 immunoreactive proteins, a marker of endothelial cells, were abundantly expressed in the LA endocardium in WKY-30W, SHR-30W, WKY-60 W, and GK-30W rats. Even during normal sinus rhythm, the immunoreactive protein level was altered differently among the SHR-30W and WKY-60W rats. As compared with the WKY-30W rats, the TFPI and TM protein expression in the LA endocardium was attenuated in SHR-30W and WKY-60W rats, respectively; however, their protein expression was preserved in the GK rats (Figure 1).

**Western blot analysis:** To quantify the protein level of TFPI and TM, a Western blot analysis was performed. CD34 immunoreactive proteins were used for an internal control. The TFPI expression was significantly reduced in both the SHR-30W (44.4% ± 24.4%) and WKY-60W (38.4% ± 19.3%) rats as compared to that in the WKY 30 W rats. On the other hand, there was no alteration in the protein level among the GK-30W rats (114.2% ± 67.4%). The TM expression was significantly reduced in the WKY-60W rats (30.1% ± 17.0%) as compared to that in the WKY-30W rats. There were no significant differences in the TM expression in the SHR-30W (40.8% ± 17.7%) and GK-30W (54.9% ± 52.94%) rats as compared to that in the WKY-30W rats (Figure 2).

**Effect of rapid atrial pacing on the antithrombotic proteins in LA endocardium:** Rapid atrial pacing has reduced the TFPI expression to 34.5% ± 21.2% (P = 0.049) in WKY-30W rats, 54.3% ± 16.3% (P = 0.018) in SHR-30W rats, 66.7% ± 27.8% (P = 0.026) in WKY-60W rats, and 48.4% ± 20.0% (P = 0.040) in GK-30W rats. Moreover, rapid atrial pacing has reduced the TM expression to 53.7% ± 15.7% (P = 0.044) in WKY-30W rats, 50.2% ± 24.2% (P = 0.010) in SHR-30W rats, 60.7% ± 33.0% (P = 0.145) in WKY-60W rats, and 65.8% ± 43.1% (P = 0.312) in GK-30W rats (Figure 3). All the rat models had a tendency to reduce the TFPI and TM expression by

### Table: Heart Weight/Body Weight and Blood Pressure Measurement

|                    | WKY 30W (n = 5) | SHR 30W (n = 5) | WKY 60W (n = 5) | GK 30W (n = 5) |
|--------------------|----------------|----------------|----------------|---------------|
| Body weight (g)    | 420 ± 9        | 392 ± 12**     | 467 ± 13*      | N/A           |
| Heart weight (mg)  | 1606 ± 46      | 1784 ± 104*    | 1870 ± 27*     | N/A           |
| H/B ratio (mg/g)   | 3.8 ± 0.2      | 4.5 ± 0.2**    | 4.0 ± 0.1      | N/A           |
| Heart rate (bpm)   | 324.3 ± 31.1   | 358.2 ± 20.9   | 316.6 ± 19.4   | 360.6 ± 30.6  |
| Systolic BP (mmHg) | 133.7 ± 15.0   | 195.3 ± 4.6*   | 135.5 ± 2.2    | 115.5 ± 11.3  |
| Diastolic BP (mmHg)| 103.7 ± 9.0    | 146.9 ± 10.7*  | 101.5 ± 4.7    | 83.8 ± 6.9    |

*P < 0.05 versus WKY 30W, **P < 0.01 versus WKY 30W, *P < 0.001 versus WKY 30W. H/B indicates heart weight/body weight.
Figure 1. Immunohistochemistry of CD31, TFPI, and TM in the left atria. CD31 immunoreactive proteins, a marker of endothelial cells, were abundantly expressed in the LA endocardium in WKY-30W, SHR-30W, WKY-60W, and GK-30W rats. TFPI and TM expression was much less in the LA endocardium of SHR-30W and WKY-60W, whereas their expression level was preserved in WKY-30W and GK-30W rats. LA indicates left atrium; TFPI, tissue factor pathway inhibitor; and TM, thrombomodulin.

Figure 2. TFPI and TM protein expression in the left atria in various rat models. The TFPI/CD34 expression was significantly reduced in the SHR-30W and WKY-60W rats as compared to that in the WKY-30W rats even during sinus rhythm. The TM/CD34 expression was significantly reduced in the WKY-60W rats as compared to that in the WKY-30W rats. TFPI indicates tissue factor pathway inhibitor; and TM, thrombomodulin.

atrial rapid pacing. No LA thrombi were identified in any of the rat groups (i.e., WKY-30W, SHR-30W, WKY-60W, and GK-30W) regardless of the rapid atrial pacing.

Discussion

The major findings of this present study were as follows: (1) Aging reduced the TM and TFPI expression, while hypertension reduced the TFPI expression in the rat atria. (2) No significant changes were noted in terms of TM and TFPI expression in DM rats. (3) Rapid atrial pacing additionally reduced the antithrombotic molecules.

The determinants of the Virchow’s triad, including blood stasis, endothelial dysfunction, and coagulation properties, are considered to be involved in atrial thrombus formation in patients with AF.6,7,10 Blood stasis due to loss of the atrial kick by fibrillating atria is one of the crucial roles of the thrombus formation in the LA appendage (LAA). However, only blood stasis might be not adequate to induce the development of an LAA thrombus in patients whose risk of thromboembolism is deemed extremely low (i.e., CHA2DS2VASc score of zero). Therefore, it is speculated that additional risk factors of endothelial dysfunction and/or the coagulation properties are involved in the mechanism of thrombus formation.

TFPI and TM are well-known antithrombotic molecules produced by the atrial endocardium for controlling the local coagulation balance.13,14 TFPI is secreted by en-
The atrial fibrillation (AF) is a common arrhythmia characterized by rapid and irregular atrial depolarization. This condition is associated with an increased risk of stroke and systemic embolism. The mechanism of AF-induced atrial remodeling is complex and involves multiple factors, including structural changes in the atrial myocardium and endocardium, as well as changes in the expression of various endogenous molecules involved in the coagulation cascade.

TFPI is a critical molecule that plays a crucial role in the regulation of the coagulation fibrinolytic systems. It binds to FXa, forming a TFPI-FXa complex that inhibits the TF-FVIIa complex. Therefore, in the presence of TFPI, it dampens the activation of clotting through an extrinsic pathway. TM is also expressed on the endocardial surface bound to thrombin, which, in turn, promotes a thrombin-mediated activation of protein C. In addition, TM and the thrombin complex inhibit the procoagulant function of thrombin. Therefore, in the presence of TFPI and TM on the vascular and myocardial endothelium, they control the normal coagulation balance preventing thrombus formation.

In this present study, TFPI and TM were noted to have already decreased in the aged rats, and TM was decreased in hypertensive rats prior to the rapid atrial pacing. However, there were no alterations in the expression levels of TFPI and TM in the DM rats. These results suggest that aging and hypertension had already impaired the LA endocardial function associated with anti-coagulation even in the absence of AF.

We have reported that rapid atrial pacing impairs the intrinsic antithrombotic function of the LA in a normal rat model. This present study confirmed that 8 hours of rapid atrial pacing uniformly reduced the expression of TFPI and TM, not only in normal rats but also in aged rats, hypertensive rats, and the DM rat model. AF occurrence augmented the impairment of the anti-coagulant barrier by decreasing the TFPI and TM expression in the rat atria. This additive effect between AF and a pathophysiological condition might accelerate the thrombus formation in the LA.

The mechanism of the impairment of the production of TFPI and TM in an aged and hypertensive rat model remains uncertain. A clinical study revealed that the endocardial TM expression in the LAA was decreased in patients with non-valvular AF. Left ventricular diastolic dysfunction, which is commonly observed in elderly and hypertensive patients, leading to atrial stretch, might be associated with endocardial dysfunction. Moreover, the blood stasis caused by AF under the presence of endothelial dysfunction might be important for the formation of LA thrombi. It is thus, reasonable to explain why hypertension and aging are identified as risk factors for strokes and systemic embolisms as shown by the CHADS2 score.

On the other hand, no alteration was observed in the TFPI and TM in the DM rat model. GK rats, which serve as mild DM model, might not be adequate to affect the endocardial remodeling of the rat atria. Different mechanisms might be involved in the clot formation in the LA. Otherwise, as shown in recent several studies, DM might not be associated with stroke and systemic thromboembolism risk.

Atrial pacing for 8 hours does not induce structural remodeling of the atria. Therefore, rapid atrial pacing-induced additive effect of downregulation of TM and TFPI was not associated with structural remodeling. In this present study, approximately 50% reduction of the TM and TFPI protein expression by rapid atrial pacing...
was observed, regardless the rat model (e.g., healthy control, hypertension, aging, and DM). Rapid atrial excitation by burst pacing might affect the protein expression. Clinical study revealed that early rhythm control was associated with the reduction of stroke and systemic embolism event rate. Therefore, additive effect to AF on endocardial dysfunction might be reversed by restoring the sinus rhythm. In this present study, atrial pacing for 8 hours showed additive effect to the various pathophysiological conditions. The short-term effect of rapid pacing could be possibly reversed; however, more than 8 hours of experiment with rats on general anesthesia using ventilator is difficult to perform due to unstable hemodynamic condition. The other large animal models will be necessary to confirm the long-term effect of atrial pacing on endothelial remodeling.

None of the rats under normal and pathophysiological conditions, even with atrial burst pacing, developed atrial thrombi in this study. It has been reported that a congestive heart failure rat model, by aortic binding followed by a myocardial ischemia reperfusion operation, revealed LA thrombi in 18.8%. However, AF was not observed in the rat model, and the relationship between AF and LA thrombi was uncertain. Nishida reported the effect of electrical and structural remodeling on atrial thrombus formation in a canine model with atrial or ventricular tachypacing, but it did not promote atrial thrombi. The incidence of stroke in patients with AF without anticoagulation therapy is extremely low especially in those with a low risk of thromboembolism, and it might be difficult to demonstrate the prothrombotic changes leading to the thrombus formation by using an animal experimental AF model.

Limitations: This study has several limitations. Firstly, this present study has focused on endocardial remodeling of the atria. Therefore, anatomical and structural data including LA diameter and interstitial fibrosis of the atria were not measured by histological analysis and echocardiography. Secondly, a recent study showed that patients with persistent or permanent AF had higher risk of stroke than those who had paroxysmal AF. Long-term atrial tachypacing might affect the endocardial dysfunction of the LA leading to thrombus formation. Thirdly, the detailed mechanism of the endocardial downregulation of TFPI and TM remains to be determined. Thus, further study will be needed to clarify these concerns. Lastly, aging, hypertension, and DM are well-known risk factors of structural remodeling of the atrial as well as that of thromboembolism. Therefore, atrial interstitial fibrosis might also affect the thrombus formation in the atrium. Diverse mechanisms are involved in the mechanism of pro-coagulation in the endothelium of the atrium.

Conclusion

Aging and hypertension, both of which are epidemiologically well-known risk factors of ischemic stroke in AF, were associated with a specific atrial endocardial dysfunction prior to AF that could additionally disturb the antithrombotic function of the atrial endocardium with atrial tachypacing.

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Disclosure

Conflicts of interest: None.

References

1. Krahn AD, Manfreda J, Tate RB, Mathewson FA, Cuddy TE. The natural history of atrial fibrillation: incidence, risk factors, and prognosis in the Manitoba follow-up study. Am J Med 1995; 98: 476-84.
2. Wolf PA, Abbott RD, Kannel WB. Atrial fibrillation as an independent risk factor for stroke: the Framingham study. Stroke 1991; 22: 983-8.
3. Gage BF, Waterman AD, Shannon W, Boechler M, Rich MW, Radford MJ. Validation of clinical classification schemes for predicting stroke: results from the National Registry of atrial fibrillation. JAMA 2001; 285: 2864-70.
4. Lip GY, Nieuwlaat R, Pisters R, Lane DA, Crijns HJ. Refining clinical risk stratification for predicting stroke and thromboembolism in atrial fibrillation using a novel risk factor-based approach: the euro heart survey on atrial fibrillation. Chest 2010; 137: 263-72.
5. Iwasaki YK, Chiba K, Kato T, Nattel S. Atrial fibrillation pathophysiology: implications for management. Circulation 2011; 124: 2264-74.
6. Ding WY, Gupta D, Lip GYH. Atrial fibrillation and the prothrombotic state: revisiting Virchow’s triad in 2020. Heart 2020; 106: 1463-8.
7. Watson T, Shantsila E, Lip GY. Mechanisms of thrombogenesis in atrial fibrillation: Virchow’s triad revisited. Lancet 2009; 373: 155-66.
8. Nishida K, Chiba K, Iwasaki YK, et al. Atrial fibrillation-associated remodeling does not promote atrial thrombus formation in canine models. Circ Arrhythm Electrophysiol 2012; 5: 1168-75.
9. Khan AA, Lip GYH. The prothrombotic state in atrial fibrillation: pathophysiological and management implications. Cardiovasc Res 2019; 115: 31-45.
10. Yamashita T, Sekiguchi A, Iwasaki YK, et al. Thrombomodulin and tissue factor pathway inhibitor in endocardium of rapidly paced rat atria. Circulation 2003; 108: 2450-2.
11. Yamashita T, Sekiguchi A, Iwasaki YK, et al. Cibenzoline attenuates upregulation of Kvl.5 channel gene expression by experimental paroxysmal atrial fibrillation. Int Heart J 2005; 46: 279-88.
12. Yamashita T, Sekiguchi A, Kato T, et al. Angiotensin type 1 receptor blockade prevents endocardial dysfunction of rapidly paced atria in rats. J Renin Angiotensin Aldosterone Syst 2007; 8: 127-32.
13. Walker FJ, Fay PJ. Regulation of blood coagulation by the protein C system. FASEB J 1992; 6: 2561-7.
14. Golino P, Ragni M, Cimmino G, Forte L. Role of tissue factor pathway inhibitor in the regulation of tissue factor-dependent blood coagulation. Cardiovasc Drug Rev 2002; 20: 67-80.
15. Ruff CT, Giugliano RP, Braunwald E, et al. Comparison of the efficacy and safety of new oral anticoagulants with warfarin in patients with atrial fibrillation: a meta-analysis of randomised trials. Lancet 2014; 383: 955-62.
16. Khan F, Tritschler T, Kahn SR, Rodger MA. Venous thromboembolism. Lancet 2021; 398: 64-77.
17. Aota T, Wada H, Yamashita Y, et al. The efficacy of the administration of recombinant human soluble thrombomodulin in pa-
tients with DIC. Int J Hematol 2016; 103: 173-9.
18. An K, Mei J, Zhu J, Tang M. Endocardial changes in nonvalvu-
lar atrial fibrillation without atrial thrombus-thrombomodulin
and tissue factor pathway inhibitor. Clin Appl Thromb Hemost
2018; 24: 1148-52.
19. Kodani E, Atarashi H, Inoue H, et al. Impact of blood pressure
control on thromboembolism and major hemorrhage in patients
with nonvalvular atrial fibrillation: a subanalysis of the J-
RHYTHM registry. J Am Heart Assoc 2016; 5: e004075.
20. Suzuki S, Yamashita T, Okumura K, et al. Incidence of ischemic
stroke in Japanese patients with atrial fibrillation not receiving
anticoagulation therapy—pooled analysis of the Shinken data-
base, J-RHYTHM Registry, and Fushimi AF registry. Circ J
2015; 79: 432-8.
21. Kirchhof P, Camm AJ, Goette A, et al. Early rhythm-control
therapy in patients with atrial fibrillation. N Engl J Med 2020;
383: 1305-16.
22. Chen J, Strauss B, Liang L, Hajjar RJ. Animal model of left
atrial thrombus in congestive heart failure in rats. Am J Physiol
Heart Circ Physiol 2019; 317: H63-72.