Enhancement of Endothelial Function Inhibits Left Atrial Thrombi Development in an Animal Model of Spontaneous Left Atrial Thrombosis

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Background: Left atrial (LA) thrombosis is an important cause of systemic embolization. The SPORTS rat model of LA thrombi (Spontaneously-Running Tokushima-Shikoku), which have a unique characteristic of high voluntary wheel running, was previously established. The aim of the present study was to investigate how SPORTS rats develop LA thrombi.

Methods and Results: Nitric oxide (NO) produced from cardiovascular endothelial cells plays an important protective role in the local regulation of blood flow, vascular tone, and platelet aggregation. No evidence of atrial fibrillation or hypercoagulability in SPORTS rats regardless of age was found; however, SPORTS rats demonstrated endothelial dysfunction and a decrease of NO production from a young age. In addition, endothelial NO synthase activity was significantly decreased in the LA and thoracic aorta endothelia of SPORTS rats. While voluntary wheel running was able to intermittently increase NO levels, running did not statistically decrease the incidence of LA thrombi at autopsy. However, L-arginine treatment significantly increased NO production and provided protection from the development of LA thrombi in SPORTS rats.

Conclusions: They present study results indicate that NO has an important role in the development of LA thrombus, and endothelia pathways could provide new targets of therapy to prevent LA thrombosis. (Circ J 2014; 78: 1980–1988)

Key Words: Atrial fibrillation; Endothelial dysfunction; Nitric oxide; Thrombosis; Vasodilation

Endothelial injury/dysfunction, along with hypercoagulability and hemodynamic changes, are characterized as Virchow’s triad, and are the major causes that contribute to thrombosis.1,2 Left atrial (LA) thrombus formation is an important cause of systemic embolization. Cardiogenic embolization, including atrial thrombosis, accounts for more than 15% of ischemic strokes,3 and approximately 30% of all patients with ischemic stroke or transient ischemic attack are found to have a potential cardiac source of embolism.4 Free-floating thrombi, separated from the left atrium, can reach peripheral tissues through blood flow and subsequently induce secondary embolisms, including cerebrovascular events. The development of LA thrombi is frequently found in patients with atrial fibrillation (AF), mitral stenosis, rheumatic heart disease, or long-term complications of the standard orthotopic heart transplantation.5,6 Investigation into thrombus formation due to AF has focused on decreased blood flow and the hypercoagulable state in the left atrium.6,7 However, although LA stasis and subsequent thrombus formation might be one source of thromboembolism, in cases of AF, this does not seem to provide a complete explanation.

Endothelial dysfunction has also been reported to play an important role in thrombosis. AF has been shown to cause atrial fibrillation; Endothelial dysfunction; Nitric oxide; Thrombosis; Vasodilation

Received November 14, 2013; accepted April 16, 2014; released online May 23, 2014  Time for primary review: 17 days
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ISSN-1346-9843  doi: 10.1253/circj.CJ-13-1398
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endothelial dysfunction leading to decreased nitric oxide (NO) levels and reduced endothelial NO synthase (eNOS) expression.10,11 NO is well known as an endothelium-derived relaxation factor, which also plays an important anti-atherosclerotic role.12 In addition, NO exerts powerful antithrombotic effects, including inhibition of platelet activation, aggregation, and adhesion to the endothelium. Therefore, reduced NO production might contribute to increased thrombogenicity.13,14

From an original Wistar strain, we established the SPORTS (Spontaneously-Running Tokushima-Shikoku) rat model. They have a unique characteristic of high voluntary wheel running,15 as well as LA thrombus formation in both male and female SPORTS rats at natural death.16 However, this model shows normal sinus rhythm and does not show AF. The aim of the present study was to investigate why LA thrombosis develops in this animal model. We investigated: (1) whether voluntary wheel running changed LA thrombus incidence in SPORTS rats; (2) whether LA thrombi were generated in young SPORTS rats; and (3) whether NO plays an important role in the development of LA thrombi in SPORTS rats. Our results reveal that this model might enable the elucidation of a detailed mechanism for LA thrombus formation and lead to new targets for therapies.

**Methods**

**Animal Preparation**

We bred male and female SPORTS rats using selective in-breeding in our laboratory, as previously described.15 SPORTS rats that ran over 10,000 revolutions on the running wheel per day (1.07 m/revolution) were selected for mating. We used male Wistar strain rats as controls (Japan SLC, Shizuoka, Japan). The rats were housed individually in temperature (23 ± 2°C) and humidity (50–60%) controlled rooms, with a dark cycle (20:00–8:00 h) and light cycle (8:00–20:00 h) daily. We administered non-purified regular chow (Oriental Yeast, Tokyo, Japan) under a light microscope (BX50F4; Olympus). Images were captured by a CCD camera (DP71; Olympus, Tokyo, Japan) under a light microscope (BX50F4; Olympus). The 2-dimensional areas of thrombus in the LA were measured using by Scion Image software (Scion Image, Frederick, MA, USA).

**Animal Study 1 – Monitoring of the Incidence of Atrial Thrombi and Life Span in Wheel Running SPORTS Rats**

To investigate the effect of voluntary wheel running on LA thrombus incidence in SPORTS rats, we divided these rats into sedentary or running groups until their natural death. The incidence of LA thrombi in SPORTS rats. We examined: (1) whether voluntary wheel running changed LA thrombus incidence in SPORTS rats; (2) whether LA thrombi were generated in young SPORTS rats; and (3) whether NO plays an important role in the development of LA thrombi in SPORTS rats. Our results reveal that this model might enable the elucidation of a detailed mechanism for LA thrombus formation and lead to new targets for therapies.

**Animal Study 2 – Monitoring of Atrial Thrombi Formation and Circulatory, Coagulation, and Metabolic Parameters**

We first compared sedentary male SPORTS vs. the normal Wistar rats as a control, which were all housed in normal cages without running wheels (n=18, Figure S1B). We examined the histological characteristics and vascular reactivity of the atria and aorta in 20-, 40-, and 60-week-old control and SPORTS rats (n=6 in each group). At 15, 30, 45, and 60 weeks of age, we measured urinary nitrite excretion (n=6). At 20 weeks of age, we measured plasma lipids and performed glucose tolerance tests (n=6), as previously reported.17 Prothrombin was assayed at 35 weeks (n=6). At 60 weeks, we monitored electrocardiogram and systolic blood pressure (n=6).

**Animal Study 3 – L-Arginine Treatment**

Male 40-week-old normal Wister rats and SPORTS rats, which were housed sedentary in normal cages, were divided into 2 treatment groups, with or without L-arginine treatment (n=6 in each group, Figure S1C). The rats were administered L-arginine dissolved in tap water (1% w/v), ad libitum for 20 weeks.

**Histological Analysis**

To observe the development of LA thrombi in SPORTS rats before their death, we harvested tissue from sedentary 20-, 40-, and 60-week-old male SPORTS rats, which were housed in individual normal cages without a running wheel. Rats that were 20, 40, or 60 weeks old were fasted for 16 h and anesthetized with an intraperitoneal injection of sodium pentobarbital (10 mg/100 g body weight) (Dainippon Sumitomo Pharma, Osaka, Japan). After anesthetization, we immediately collected blood and tissue samples. The hearts were cut in half and rinsed well with saline to remove residual blood. The isolated tissues were fixed in 4% buffered p-formaldehyde for 48 h and embedded in paraffin, then cut to 3 μm in thickness and stained with hematoxylin-eosin or phosphotungstic acid hematoxylin. Images were captured by a CCD camera (DPT1; Olympus, Tokyo, Japan) under a light microscope (BX50F4; Olympus). The 2-dimensional areas of thrombus in the LA were measured using by Scion Image software (Scion Image, Frederick, MA, USA).

**Vascular Reactivity**

Vascular reactivity of thoracic aortas was measured, as previously described.20 Thoracic aortas were removed and placed in cold Krebs-Ringer bicarbonate solution (KRB) containing (in g/L): NaCl 6.92, NaHCO3 2.10, D-glucose 2.00, KH2PO4 0.16, MgSO4-7H2O 0.29, KCl 0.35, and CaCl2 0.28. Residual blood and perivascular tissue was removed in cold KRB, and the aortas were cut into ring segments of 3 mm length. Each ring was then placed in a 3 ml organ bath (Micro Easy Magus; Kishimoto Medical, Kyoto, Japan) and mounted on 2 stainless steel wires, one of which was fastened to the bath and the other connected to a force transducer for the measurement of isometric tension. The bath was filled with KRB solution at 37°C and bubbled with a mixture of 95% O2–5% CO2. The vasodilative effects by cumulative concentration of acetylcholine (Ach) or sodium nitroprusside (SNP) were measured after vasoreactions, which was induced by 100 μmol/L phenylephrine (PE).

**Tissue Preparation and Immunoblotting**

Left atrial and aortic tissues were rapidly isolated from anesthetized rats and residual blood, and adherent tissue was removed in cold phosphate-buffered saline. Tissues were stored at −80°C until the evaluation by immunoblot analyses, as pre-
Slightly lower rates of LA thrombi than sedentary groups, although the difference was not statistically significant (male rats, \( P=0.60 \); female rats, \( P=0.19 \)).

**LA Thrombi Were Found in the Early Stage of Life in SPORTS Rats**

At 20 weeks of age, there was no significant difference in the heart weight between control and SPORTS rats; however, the heart weights of SPORTS rats at 40 and 60 weeks were significantly greater than the corresponding age-matched controls (Table S1). In the atria of 20- and 40-week-old rats, a histological examination showed small thrombi in the LA of SPORTS rats but not in control rats (Figure 2). Furthermore, larger-sized thrombi were found in the LA of 60-week-old SPORTS rats (Figures 2H, I). These thrombi consisted of reticulated strands of fibrin and aggregation of red blood cells and platelets (Figures 2C, F, I), which is similar to the histological findings of models of LA thrombosis. And SPORTS rats had some obvious thickened and injured endothelium in their LA (red arrows in Figures 2F, I). Control rats did not have thrombi in the LA at any age (Figures 2A, D, G). These results suggest that LA thrombi are generated starting from early stages of life in SPORTS rats.

**SPORTS Rats Did Not Reveal AF or Hypercoagulability**

Cardiac dysrhythmia, especially AF, is well known as one of the major cardiogenic causes for atrial thrombosis. Electrocardiography, monitored by a telemetry transmitter system, did not detect any arrhythmias in SPORTS rats (Figure S2), suggesting that LA thrombi in SPORTS rats were not due to AF. In addition, heart rates were not significantly different be-

| Table 1. Incidence of Atrial Thrombi and Lifespan in SPORTS Rats With and Without Exercise |
|------------------------------------------|---------------------------------|---------------------------------|------------------|
|                                          | AT n (%) | Non-AT n (%) | Total n | Lifespan (weeks) |
|------------------------------------------|----------|--------------|---------|------------------|
| **Male SPORTS**                          |          |              |         |                  |
| Sedentary                                | 3 (60)   | 2 (40)       | 5       | 79.7±14.4        |
| Running                                  | 14 (54)  | 12 (46)      | 26      | 80.6±28.2        |
| **Female SPORTS**                        |          |              |         |                  |
| Sedentary                                | 7 (50)   | 7 (50)       | 14      | 117.9±25.2       |
| Running                                  | 12 (35)  | 22 (65)      | 34      | 96.6±27.5        |

Male and female SPORTS rats were divided into 2 groups: sedentary groups housed in normal cages, or voluntary running groups housed in cages with a running wheel. Values for the lifespan are shown as mean(weeks)±SD. AT, atrial thrombus in the left atrium; SPORTS, Spontaneously-Running Tokushima-Shikoku.

Reagents

PE hydrochloride, Ach chloride, L-arginine, and the assay kits for triglycerides (TG), total cholesterol, and high-density lipoprotein (HDL) cholesterol were purchased from Wako Chemicals (Tokyo, Japan). Antibodies against ENOS were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody against inducible NOS (iNOS) was purchased from BD Transduction Laboratories (Lexington, KY, USA). Antibodies against glyceraldehyde 3-phosphate dehydrogenase and \( \alpha \)-smooth muscle actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Statistical Analysis**

Statistical analysis of differences was calculated using ANOVA plus Bonferroni multiple comparison tests. A value of \( P<0.05 \) was considered statistically significant. The incidence of LA thrombi, which had 2 categorical variables, was analyzed using the chi-squared test of independence or Fisher’s exact probability test.

**Results**

**Voluntary Running Had Little Effect on the Incidence of LA Thrombi in SPORTS Rats**

The average life spans were not significantly different between the sedentary and running groups (Table 1). The 36 to monitored 79 SPORTS rats had some thrombi in the LA (Table 1; Figure 1). In both male and female rats, running groups showed slightly lower rates of LA thrombi than sedentary groups, although the difference was not statistically significant (male rats, \( P=0.60 \); female rats, \( P=0.19 \)).

**LA Thrombi Were Found in the Early Stage of Life in SPORTS Rats**

At 20 weeks of age, there was no significant difference in the heart weight between control and SPORTS rats; however, the heart weights of SPORTS rats at 40 and 60 weeks were significantly greater than the corresponding age-matched controls (Table S1). In the atria of 20- and 40-week-old rats, a histological examination showed small thrombi in the LA of SPORTS rats but not in control rats (Figure 2). Furthermore, larger-sized thrombi were found in the LA of 60-week-old SPORTS rats (Figures 2H, I). These thrombi consisted of reticulated strands of fibrin and aggregation of red blood cells and platelets (Figures 2C, F, I), which is similar to the histological findings of models of LA thrombosis. And SPORTS rats had some obvious thickened and injured endothelium in their LA (red arrows in Figures 2F, I). Control rats did not have thrombi in the LA at any age (Figures 2A, D, G). These results suggest that LA thrombi are generated starting from early stages of life in SPORTS rats.
Endothelial Dysfunction in an Atrial Thrombosis Model

SPORTS Rats Showed Endothelial Dysfunction in the Thoracic Aorta

Thrombus formation has been closely associated with endothelial dysfunction. Vascular endothelial cells have important roles in homeostasis, such as regulation of vascular tone, platelet aggregation, and blood coagulation. Compared to control rats, 20-week-old SPORTS rats showed thickened neointima and thrombus formation in the thoracic aortas (Figures 3A, B). To observe endothelium-dependent vasodilation, we measured the vascular reactivity of isolated thoracic aortas from 20-week-old rats (Figures 3C, D). Compared with controls, SPORTS rats showed decreased Ach-induced vasodilation response (Figure 3C); however, the vasodilative effect of the NO donor, SNP, was not different between control and SPORTS aortas. Therefore, SPORTS rats had decreased endothelium-derived vasodilation. Similarly, endothelium-dependent relaxation was decreased in 40- and 60-week-old SPORTS rats (data not shown). These results suggest that SPORTS rats had endothelial dysfunction in the thoracic aorta.

SPORTS Rats Have Normal Plasma Lipids and Glucose Tolerance

Because thrombus formation is often associated with lifestyle-related diseases such as hyperlipidemia, hypercholesterolemia, and diabetes mellitus, we examined plasma lipids and glucose tolerance in SPORTS vs. control rats (Table 2). Although significant differences were not found in the fasting levels of total and HDL cholesterol and insulin, the levels of fasting plasma TG and glucose in SPORTS rats were significantly lower than those of control rats. Cumulative levels (area under the curve) of plasma glucose and insulin during the glucose tolerance test were not different between control and SPORTS rats. These results suggest that development of LA thrombi in SPORTS rats was not due to a disorder of plasma lipids and glucose tolerance.
SPORTS Rats Have Downregulated eNOS in the Left Atrium and Thoracic Aorta

We hypothesized that the development of LA thrombi in SPORTS rats was due to a decrease of NO production, mediated via NO synthase (NOS). Three isoforms of NOS have been identified from independent genes: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). Several recent reports have demonstrated that eNOS downregulation in the LA was associated with the development of LA thrombi formation. In both the LA and aortas, SPORTS rat eNOS expression was significantly decreased compared to tissues from control rats, although there was no difference in iNOS expression between control and SPORTS rats (Figure 4). Furthermore, urinary NO2 excretion from sedentary SPORTS rats was significantly lower than that of control rats, consistent from 15 to 60 weeks of age (Figure 5). From these results, we conclude that the decrease of NO production in SPORTS rats is due to the downregulation of eNOS expression, possibly explaining the development of LA thrombi in the SPORTS model.

L-Arginine Treatment Increased NO Production and Decreased the Development of LA Thrombi

NO is synthesized from L-arginine as an enzymatic substrate of NOS. L-arginine treatment increases NO production and has protective effects for the local regulation of blood flow, vascular tension, and platelet aggregation of endothelial cells. To observe the effect of L-arginine treatment on NO produc-
Endothelial Dysfunction in an Atrial Thrombosis Model

In SPORTS rats, the bi-section area of the left atrium was significantly decreased by L-arginine treatment (Figure 6B). From these results, we suggest that the development of LA thrombi is due to a decrease of NO production, and that L-arginine treatment provides protection against LA thrombosis.

In addition, we measured urinary NO3 excretion. Treatment with L-arginine significantly increased urinary NO3 in SPORTS rats during both dark and light periods (Figure 6A). Next, we investigated the effect of L-arginine treatment on the development of LA thrombi. The 2-dimensional area of the LA thrombi section in SPORTS rats was significantly decreased by L-arginine treatment (Figure 6B). From these results, we suggest that the development of LA thrombi is due to a decrease of NO production, and that L-arginine treatment provides protection against LA thrombosis.
Cardiac dysrhythmia, especially AF, is a well-known cause of LA thrombus formation. In the present study, we found LA thrombi in young as well as adult SPORTS rats, without AF or hypercoagulability. SPORTS rats had endothelial dysfunction and decreased NO production from a young age. In addition, eNOS expression was significantly decreased in the LA and thoracic aortas of SPORTS rats. NO produced from endothelial cells plays important protective roles in the local regulation of blood flow, vascular tone, and platelet aggregation. From these data, we suggest that the development of LA thrombi might, at least in part, be due to decreased NO production. Furthermore, we found that L-arginine treatment significantly increased NO production and had protective effects for the development of LA thrombus in SPORTS rats. Recent reports found that LA thrombi formation induced by AF might have a marked decrease in eNOS expression and NO bioavailability in the LA. Our results indicate that NO has an important role in the development of LA thrombus, regardless of the fibrillation state of the atria.

In this study, we had just focused on relatively old thrombi in the left atrium. Old thrombi, which have a rigid structure, are highly organized, mostly by collagen in the perpetuation stage. In contrast, fresh thrombi, which are still in the development and expansion stage, consist mainly of blood material and are not yet organized by collagen. These fresh thrombi are more elastic and have a lower stiffness, therefore, we immediately cut the hearts in half and rinsed them well with saline to removing residual blood before fixation. In the vasculature, endothelial dysfunction is also known to play an important role in thrombus development, including by decreasing blood flow and hypercoagulability, as shown in the well-known Virchow’s triad. Recent experimental and clinical studies have demonstrated that Virchow’s triad is also important in thrombus formation in the fibrillating left atrium. Obvious thickened and injured endothelium of the LA surface in SPORTS rats was observed by histological analyses (Figures 2F, I), von Willebrand factor (vWF), a well-established index of endothelial damage and dysfunction, associate to the platelets via the matrix formation with glycoprotein Ib/IX. vWF was significantly increased in the injured atrial endocardium containing inflammatory cells in patients with AF who have a history of cardioembolic thromboembolism. From these reports, vWF might be a key factor of LA thrombosis in SPORTS rats. Several recent reports have demonstrated that AF, per se, causes atrial endocardial dysfunction, which is manifested by increased plasminogen activator inhibitor-1 expression, decreased expression of eNOS, tissue factor pathway inhibitor, and thrombomodulin in the LA endocardium, leading to local coagulation imbalance on the internal surface of the atrium. Another report reported that inflammation might not only result in endothelial damage and dysfunction but might also be linked directly to thrombogenesis. Our previous study indicated that many neutrophils accumulated around each thrombus, and some neutrophils and lymphocytes were observed to infiltrate the atrial wall in SPORTS rats. Interleukin 6, a cytokine produced from these inflammatory cells, increases sensitivity to thrombin and stimulates transcription of fibrinogen. These reports suggest that the imbalance of coagulation on the internal surface of the atrium is an important factor for atrial thrombus formation.

Discussion

Cardiac dysrhythmia, especially AF, is a well-known cause of LA thrombus formation. In the present study, we found LA thrombi in young as well as adult SPORTS rats, without AF or hypercoagulability. SPORTS rats had endothelial dysfunction and decreased NO production from a young age. In addition, eNOS expression was significantly decreased in the LA and thoracic aortas of SPORTS rats. NO produced from endothelial cells plays important protective roles in the local regulation of blood flow, vascular tone, and platelet aggregation. From these data, we suggest that the development of LA thrombi might, at least in part, be due to decreased NO production. Furthermore, we found that L-arginine treatment significantly increased NO production and had protective effects for the development of LA thrombus in SPORTS rats. Recent reports found that LA thrombi formation induced by AF might have a marked decrease in eNOS expression and NO bioavailability in the LA. Our results indicate that NO has an important role in the development of LA thrombus, regardless of the fibrillation state of the atria.

In this study, we had just focused on relatively old thrombi in the left atrium. Old thrombi, which have a rigid structure, are highly organized, mostly by collagen in the perpetuation stage. In contrast, fresh thrombi, which are still in the development and expansion stage, consist mainly of blood material and are not yet organized by collagen. These fresh thrombi are more elastic and have a lower stiffness, therefore, we immediately cut the hearts in half and rinsed them well with saline to removing residual blood before fixation. In the vasculature, endothelial dysfunction is also known to play an important role in thrombus development, including by decreasing blood flow and hypercoagulability, as shown in the well-known Virchow’s triad. Recent experimental and clinical studies have demonstrated that Virchow’s triad is also important in thrombus formation in the fibrillating left atrium.
of reports about thrombosis in the LA is higher than that for RA. Furthermore, in an AF model and patients, endothelial dysfunction, containing a decrease in NO production or an increase in vWF expression, was observed only in the LA and not in the RA. From these reports, LA might be easy to suffer endothelial dysfunction and thrombosis rather than RA. Formation of LA thrombi is known to be an important risk factor for cerebral or the other embolism. In the progress study, our collaborators observed paralytic symptoms in some SPORTS rats, but a question that depends on thromboembolism in the brain or not remains unclear. In addition, one of main causes of death in SPORTS rats seemed to be heart failure, but the actual numbers of each cause of death were not monitored in this study. Our collaborators observed that both atrial natriuretic peptide and brain natriuretic peptide were increased in the heart tissue of SPORTS rat (unpublished data), but it remains unclear why SPORTS rats cause heart failure. We thought that two of the factors was a slightly higher blood pressure and heart rate than control rats (Table 2), which might be caused by a high catecholamine condition.

More detailed analyses of the heart, brain, and other tissues in SPORTS rats are necessary in the progress study.

In this study, we established a new model of LA thrombosis with decreased NO production, but without AF. Adrie et al reported in an animal study that inhaled NO might inhibit the development of coronary thrombosis. Another report found that plasma NO levels were significantly increased after exercise compared with that of sedentary rats. In addition, there is increasing evidence that regular exercise reduces the risk of arterial and venous thrombotic events, due to reduced circulating levels of viscosity factors and inflammatory variables.

In our model, voluntary wheel running increased NO levels inSPORTS rat, but could not significantly decrease the incidence of LA thrombi. From these results, the type of exercise to constantly increase NO levels might be an important factor in preventing arterial thrombosis. Subsequently, we found that L-arginine treatment significantly increased NO production and efficiently inhibited the development of LA thrombus. Our data were supported by other reports that found the supplement of L-arginine to ameliorate the endothelial dysfunction and thrombus formation in models of both arterial and venous thrombosis.

Hydroxymethylglutaryl (HMG-CoA) reductase inhibitors and omega-3 fatty acids, which are known to improve endothelial dysfunction, show the protective effect in animal models of AF, but the results, which have the effectiveness or not, remain controversial in AF patients.

Therefore, it is still unknown which therapies focused on endothelial dysfunction alone, and which therapies were used that were similar to this study that could recover LA thrombosis completely in AF patients. Shin et al reported that catheter ablation for AF patients with no recurrence improved endothelial dysfunction by the measurement of flow-mediated dilation (FMD), but they needed for 6 months to the FMD score of control subject. Furthermore, the patients with recurrence continue endothelial dysfunction after AF ablation.

However, some reports showed that HMG-CoA reductase inhibitors and omega-3 fatty acids had protective effects for AF patients post-cardioversion or ablation.

We thought that the animal model without AF we used in this study is similar to that of patients post-cardioversion or ablation, and some continuous anticoagulant therapies or treatment for endothelial dysfunction might be beneficial in these patients without an AF condition. The results of the present study provide details towards deciphering a new model of LA thrombosis, as well as a new direction towards targets of therapy to prevent LA thrombosis.

Acknowledgments

This study was supported, in part, by the Grant-in-Aid for Scientific Research to Y. Nakaya (No. 19300222) and to K. Mawatari (No. 24500857) from the Ministry of Education, Science, Sports, and Culture and Technology, Japan.

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