Downregulation of Endocardial Nitric Oxide Synthase Expression and Nitric Oxide Production in Atrial Fibrillation

Potential Mechanisms for Atrial Thrombosis and Stroke

Hua Cai, MD, PhD; Zongming Li, MD; Andreas Goette, MD; Fernando Mera, MD; Clegg Honeycutt, MS; Kristian Feterik, MD; Josiah N. Wilcox, PhD; Samuel C. Dudley, Jr, MD, PhD; David G. Harrison, MD; Jonathan J. Langberg, MD

Background—In the arterial endothelium, laminar flow and cyclical stretch induce expression of NO synthase (NOS). We hypothesized that atrial fibrillation (AF) causes a downregulation of atrial endocardial NOS expression and NO production. Because NO has antithrombotic properties, this may contribute to thromboembolism in AF.

Methods and Results—In pigs, AF was produced with rapid atrial pacing at 600 bpm for 1 week, whereas controls had atrial pacing at 100 bpm. All animals had catheter ablation of the AV junction and ventricular pacing at 100 bpm. NO production from freshly isolated tissue was measured by a NO-specific microelectrode. Left atrial basal and stimulated NO production was decreased in AF by 73% and 71% \((P<0.01)\). Endocardial NOS expression, determined by Western analysis, was also significantly decreased by 46%. Expression of the prothrombotic protein plasminogen activator inhibitor 1 (PAI-1) is known to be regulated by NO and was increased in the left atrium by 1.8-fold in AF \((P<0.05)\). NO concentration was decreased in the left atrial appendage, although NOS expression was not affected. Neither NOS concentration, NO levels, nor PAI-1 expression were altered in the aortas or right atria of animals with AF.

Conclusions—AF is associated with a marked decrease in endocardial NOS expression and NO bioavailability and an increase in PAI-1 expression in the left atrium. These data suggest that organized atrial contraction is needed to maintain normal endocardial expression of NOS. It is likely that loss of this antithrombotic enzyme contributes to the thromboembolic phenomena commonly observed in AF. (Circulation. 2002;106:2854-2858.)

Key Words: nitric oxide ■ arrhythmia ■ thrombosis ■ fibrillation ■ plasminogen activators

Atrial fibrillation (AF) is the most common cardiac arrhythmia, with a prevalence that doubles with every decade of life, exceeding 5% in people over 70 years of age.1 Atrial fibrillation is associated with a 5- to 6-fold increase in the incidence of stroke, attributable almost exclusively to thrombus formation in the left atrial appendage. Recently, it has been found that atrial fibrillation produces a hypercoagulable state.2–6 The mechanisms responsible for this hypercoagulable state remain undefined.

See p 2764

Nitric oxide (NO) exerts powerful antithrombotic effects in arterial endothelium. Early studies demonstrated that NO inhibits platelet activation.7,8 It has been shown recently that NO released from activated platelets inhibits platelet recruitment to the growing thrombus.9 The antithrombotic effects of NO on arterial endothelium are also attributable to inhibition of the expression of the prothrombotic protein plasminogen activator inhibitor-1 (PAI-1).10,11 Nitric oxide is synthesized by nitric oxide synthase (NOS), which is present in large concentrations in the endothelium. In the endothelium, it has been shown that NOS expression is regulated by flow-mediated shear stress and is downregulated at sites with low flow.12–15 Because AF leads to a loss of organized atrial contraction and to turbulent blood flow in the left atrium, we hypothesized that AF may cause a downregulation of NOS gene expression and function in the left atrial endocardium. The subsequent decrease in NO concentration may lead to increased expression of PAI-1 and platelet aggregation and ultimately contribute to thrombosis in the left atrium.

Methods

Porcine Model of Atrial Fibrillation

Experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at Emory University. Fourteen pigs of either sex weighing 25±4 kg were studied.
Experimental Group – AV block, rapid atrial pacing, ventricular pacing at 100 BPM

Control Group – AV block, dual chamber pacing at 100 BPM

Figure 1. Diagram of pacing methods used in the experimental and control groups along with representative ECGs.

Anesthesia was induced with ketamine (20 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg) intramuscularly and maintained with isoflurane 1.5% to 2%.

The right internal jugular vein was isolated, and a tined, bipolar permanent pacing lead was introduced, advanced under fluoroscopic guidance, and positioned in the apex of the right ventricle. An active fixation bipolar lead was introduced into the left internal jugular vein using comparable technique and placed in the right atrial appendage. All animals had complete AV block produced by catheter ablation (see below). In the study group (n=7), the atrial lead was attached to a Xyrel neurostimulator (Medtronic) that was positioned subcutaneously in the neck. The device was programmed to a rate of 600 bpm to maintain atrial fibrillation. A separate single-chamber pacemaker was attached to the ventricular lead and programmed to a rate of 100 bpm. In the control group, the atrial and ventricular leads were connected to a single DDD pacemaker programmed to rate of 100 bpm and an AV delay of 120 ms. In this way, both the experimental and control animals had a paced ventricular rate of 100 bpm and differed only in their atrial rhythm (Figure 1).

After the pacemakers had been implanted, the animals underwent catheter ablation of the atrioventricular junction. A hemostatic sheath was inserted percutaneously into the right femoral vein. A radiofrequency ablation catheter (EP Technologies Stereocath) was introduced via the sheath and advanced to the tricuspid annulus. It was positioned to record a His bundle potential, and a motorized micromanipulator. For each experiment, 3 measurements for each tissue area were averaged. The order of tissue segments studied was varied. NO-dependent oxidation currents were recorded (voltage clamp mode) using an Axopatch 200B amplifier (Axon Instruments). Recordings were made at 0.7 V, filtered at 2 Hz, and digitized at 500 Hz. pCLAMP 7.0 (Axon Instruments) was used to deliver voltage protocols and acquire and analyze data.

Measurement of Endocardial NOS and PAI-1 Protein Levels

Freshly isolated cardiac and aortic tissue samples were homogenized on ice with a Tissue Tearor (Biospec Products Inc) in homogenization buffer (50 mmol/L Tris, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Triton, 1 mmol/L PMSF, 1 mmol/L leupeptin, 1× protease inhibitor cocktail from Roche Diagnostics). The protein concentration in the Triton-soluble supernatant was determined using the Lowry Assay. Equal amounts of protein samples (45 μg) were separated in 7.5% to 10% SDS-PAGE. Monoclonal anti-NOS (1:1000) and anti-PAI-1 (1:2000) antibodies (BD Transduction Laboratories, Lexington, Ky) were used to detect NOS and PAI-1 protein expression, respectively. The densitometry of the NOS and PAI-1 bands were performed with a Gel Doc 1000 system (Bio-Rad Laboratories).

Immunohistochemical Staining of Endocardial NOS

Immunohistochemistry of NOS was performed as described previously.17 In brief, frozen, paraformaldehyde-fixed tissue sections were thawed and fixed in acetone for 5 minutes, dried, and rehydrated in PBS. The primary antibody against NOS (H-32 raised against bovine NOS from BIOIMOL Research Laboratories, Plymouth Meeting, Pa) at a titer of 1:400 in 1.0% BSA in PBS was applied and incubated in a humidified chamber for 60 minutes at room temperature. The sections were washed in PBS and then incubated with a secondary antibody (biotinylated horse anti-mouse antibody from Vector Laboratories, Burlingame, CA) also at a titer of 1:400 in PBS containing 1.0% BSA and 2.0% normal horse serum for 30 minutes at room temperature. This was followed by washing the sections in PBS and incubation with an avidin-biotin enzyme complex and chromogenic substrate as described by the manufacturer. NOS proteins were visualized using the Vectastain Elite ABC peroxidase system or the Vectastain ABC alkaline phosphatase system with substrate kit III (blue reaction product; Vector Laboratories). Serial sections treated with secondary antibodies only or with nonimmune IgG did not show any staining.

Statistical Analysis

Data are presented as mean±1 SEM. Comparisons between experimental and control animals were performed using Student’s t test for unpaired data. P<0.05 was considered statistically significant.

Results

At the time of euthanasia, an ECG confirmed the presence of AF in each of the experimental animals and an atrial paced rhythm in each of the controls. Complete atrioventricular block was persistent in all animals, as was a ventricular paced rhythm at 100 bpm. There was no difference in blood pressure between the
experimental and control groups (systolic $112\pm 23$ mm Hg versus $108\pm 32$ mm Hg, $P=NS$). At the time of euthanasia, no atrial thrombi were seen on gross inspection.

**Endocardial NO Concentration Is Decreased in AF**

NO concentration was measured under basal conditions and after stimulation with the calcium ionophore A23187 ($1\mu$mol/L). In the control animals, basal left atrial NO concentration was at least 3-fold higher than in the aortic endothelium or in the ventricular endocardium. NO concentration was 3.7 times less in left atria that had been subjected to AF for 1 week compared with control left atria ($15\pm 6$ versus $56\pm 16$ nmol/L, $P<0.01$, Figure 2). AF also dramatically decreased stimulated left atrial NO concentration ($31\pm 12$ nmol/L versus $107\pm 34$ nmol/L for control left atria, $P<0.01$, Figure 3). The effects of AF on NO production were comparable in the left atrial appendage (Figures 2 and 3). AF did not cause a significant change in either basal or stimulated NO release in the ascending aorta compared with control specimens.

**Downregulation of Endocardial NOS Expression in AF**

Endocardial NOS expression from AF and control animals was quantified by Western analysis using a monoclonal antibody against NOS. NOS protein expression in the left atria of the AF group was 46% less than in control animals ($4.69\pm 0.64$ versus $8.72\pm 1.59$ densitometric units, $P<0.01$, Figure 4). Immunohistochemical staining showed that most NOS activity was endocardial and that differences between the groups were confined to this region (Figure 5). Left ventricular NOS levels were significantly decreased in animals with AF compared with controls, although this difference was not as pronounced as in the left atria. In the right atria and ventricle, there was a similar trend that did not achieve statistical significance (Figure 4). NOS protein expression was not different in the aortas of control and experimental animals, consistent with the observation that aortic NO concentrations were similar in the two groups.

Surprisingly, there was no significant difference in NOS expression between control and AF animals in the left atrial appendage (Figure 6), despite the observation that NO concentration was decreased in the appendage in the AF group. The immunohistochemical staining of NOS in the atrial appendages was highly heterogenous, corresponding to the heavily trabeculated endocardial surface.

**Upregulation of Endocardial PAI-1 Expression in AF**

In arterial endothelium, NO levels have been shown to be inversely related to the prothrombotic protein PAI-1. To determine if this reciprocal relationship was also present in the atrial endocardium, PAI-1 protein levels were determined by
There was, however, no significant increase in PAI-1 expression differed from those of the body of the left atrium. Although the right atrium or aorta. Findings in the left atrial appendage densitometric units for control versus AF, $P_{\text{mean}}$ SEM. * /H11006 bottom panels are grouped densitometric data presented as mean±SEM.

Western analysis using a monoclonal antibody. The expression of PAI-1 in the left atrium was increased by 1.8-fold in the AF animals compared with controls (6.88±1.36 versus 3.92±0.53 densitometric units for control versus AF, $P<0.05$, Figure 7). There was, however, no significant increase in PAI-1 expression in either the atrial appendages or the aorta (Figure 7).

**Discussion**

In a porcine model, we have observed that AF induces endocardial dysfunction, particularly in the left atrium. AF caused a marked decrease in endocardial NOS expression and a corresponding drop in NO concentration measured at the endocardial surface. These changes were associated with an increase in PAI-1 expression in the left atrium. AF did not significantly change NO concentration or NOS expression in the right atrium or aorta. Findings in the left atrial appendage differed from those of the body of the left atrium. Although AF was associated with a marked reduction in NO levels, it did not cause a decrease in NOS.

This is the first study to show that the left atrium produces 3-fold more NO than other endothelium and to localize the AF-induced NO reduction to the left atrial endocardium, the site of thrombus formation in these patients. It is interesting to note that control levels of NO were less in the right atrium than the left atrium and that AF had less of an effect on NO levels on the right side. This is consistent with the observation that venous NOS expression is less than that in arterial endothelium.

Mechanical stimuli have been shown to influence several aspects of endothelial function. The constant shear stress produced by unidirectional flow maintains normal endothelial NOS protein (eNOS) expression by activation of the tyrosine kinase c-Src, which then leads to divergent pathways modulating both eNOS transcription rate and also its mRNA stability. Shear stress also acutely increases NO bioavailability in the endothelium by stimulating eNOS phosphorylation. NO is known to inhibit many aspects of atherosclerosis, such as platelet aggregation, inflammatory adhesion molecule expression, and vascular smooth muscle cell proliferation. Therefore, it has been suggested that loss of NOS expression may contribute to lesion formation at sites of disturbed flow. An increase in PAI-1 attributable to a loss of NO at these sites may also contribute to lesion formation. Some of the PAI-1 expressed and released by endothelial cells remains associated with the cell surface. It can reduce endocardial fibrinolytic activity, thus promoting local thrombus formation. Increased levels of PAI-1 have been identified in atherosclerotic human arteries, in neointima formed after vein graft failure, and in balloon-injured blood vessels. Although it is interesting to speculate that the downregulation of NO production caused the increase in PAI-1, our present studies do not prove this. Nevertheless, the elevation of PAI-1 we observed in atria with AF may contribute to thrombosis in this condition.

An interesting finding in the current study is that the left atrial production of NO, as determined by the NO-specific electrode, was two to three times higher than that observed in the arterial endothelium. It has been proposed that nitrosothiols formed by the reaction of NO with compounds like albumin, glutathione, or hemoglobin may serve as circulating forms of NO, affecting vascular tone and thrombosis throughout the circulation. Given the very high levels of NO produced by the left atrium and the fact that the entire cardiac output passes through this chamber during each cardiac cycle, it is possible that it is an important source of such circulating compounds. Thus, the atrial endocardium may serve as an endocrine organ, releasing nitroso compounds into the circulation to modulate vascular function and thrombosis throughout the circulation. Indeed, Minamino et al have reported that plasma levels of nitrite/nitrate and platelet cGMP levels are decreased in patients with AF. They speculated that this was attributable to turbulent flow during AF causing arterial endothelial dysfunction. The results of our study suggest that at least some of the changes in plasma NOx are attributable to decreased production of NO in the left atrium. An additional study comparing AF animals with intact AV conduction to those with AV block and ventricular pacing would elucidate the contribution of the arterial endothelium to the systemic thrombotic state seen in AF.

To our knowledge, the present study is the first report of left atrial endocardial dysfunction in AF, manifest as a decrease in NO bioavailability. In a study of 52 patients with permanent AF, Li-Saw-Hee et al found elevation of von Willebrand factor and soluble thrombomodulin compared with control patients. The recent report from the Framingham Offspring Study also found...
that 47 subjects with AF had higher levels of fibrinogen, von Willebrand factor, and tissue-type plasminogen activator antigen than those without AF.6 Patients with AF have elevation of β-thromboglobulin and platelet factor 4.3,4 The soluble adhesion molecule P-selectin is also increased in AF, consistent with platelet activation in these patients. The coagulation cascade is also abnormally activated. Fibrinogen, PAI-1 concentration, and thrombin-antithrombin III complex levels are elevated in patients with AF compared with matched controls.2,6,25,26 It is interesting to note that these abnormalities do not develop immediately after the onset of AF. Evidence of activation of fibrinoenucleation is first detectable 12 hours after onset of the arrhythmia and increases progressively with the duration of the episode.4 This may be why the risk of stroke is low with episodes of AF that last less than 1 or 2 days. The delay between the onset of AF and hypercoagulability is consistent with the observations in the present study showing that AF induces endocardial dysfunction, because the downregulation of NOS and its associated effects take time to develop.

The expression of NOS in the left atrial appendage in the AF group was not different from that in the control animals, despite the fact that endocardial NO concentration is reduced at this site. There are several possible explanations for this discordance. It is possible that myocarcial and capillary endothelial NOS contributed to the discordance. Alternatively, it may be that decreased NO bioavailability is caused by factors that affect NOS enzyme activity or NO stability rather than direct consequences of altered NOS expression. Recent studies suggest that AF is associated with increased atrial oxidant stress, which is known to hasten inactivation of NO by superoxide.27,28 It is possible that the highly heterogeneous distribution of NOS in the left atrial appendage made the overall examination of NOS protein expression by Western analysis insensitive. This very patchy expression of NOS in the left atrial appendage was also seen in a study of patients with atrial fibrillation.29 Interestingly, adherent platelets were seen only in sections of the endocardium that were deficient in NOS.

In summary, AF is associated with a marked decrease in NOS expression and NO bioavailability as well as a significant increase in PAI-1 expression in left atrium. These data indicate that laminar blood flow in sinus rhythm and the cyclic stretch of atrial endocardial cells may act as a stimulus to maintain normal endocardial expression and function of NOS. It is likely that loss of this important antithrombotic enzyme contributes to the thromboembolic phenomena commonly observed in AF.

Acknowledgments

This study was supported by National Institutes of Health grants HL39006 (to Dr Harrison), HL64826 (to Dr Dudley), and HL59248 (to Dr Dudley), National Institutes of Health Project Grant 58000 (to Dr Harrison), a Department of Veterans Affairs merit grant (to Dr Harrison), a Procter and Gamble University Exploratory Research Grant (to Dr Dudley), a Scientist Development Award from the American Heart Association (to Dr Dudley), and a Postdoctoral Fellowship Award from the American Heart Association (to Dr Cai).

References

Downregulation of Endocardial Nitric Oxide Synthase Expression and Nitric Oxide Production in Atrial Fibrillation: Potential Mechanisms for Atrial Thrombosis and Stroke
Hua Cai, Zongming Li, Andreas Goette, Fernando Mera, Clegg Honeycutt, Kristian Feterik, Josiah N. Wilcox, Samuel C. Dudley, Jr, David G. Harrison and Jonathan J. Langberg

Circulation. 2002;106:2854-2858; originally published online November 11, 2002;
doi: 10.1161/01.CIR.0000039327.11661.16
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/106/22/2854

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/