Atrial Fibrillation Increases Production of Superoxide by the Left Atrium and Left Atrial Appendage
Role of the NADPH and Xanthine Oxidases

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Background—Atrial fibrillation (AF) is associated with an increased risk of stroke due almost exclusively to emboli from left atrial appendage (LAA) thrombi. Recently, we reported that AF was associated with endocardial dysfunction, limited to the left atrium (LA) and LAA and manifest as reduced nitric oxide (NO) production and increased expression of plasminogen activator inhibitor-1. We hypothesized that reduced LAA NO levels observed in AF may be associated with increased superoxide (O$_2^-$) production.

Methods and Results—After a week of AF induced by rapid atrial pacing in pigs, O$_2^-$ production from acutely isolated heart tissue was measured by 2 independent techniques, electron spin resonance and superoxide dismutase–inhibitable cytochrome C reduction assays. Compared with control animals with equivalent ventricular heart rates, basal O$_2^-$ production was increased 2.7-fold (P<0.01) and 3.0-fold (P<0.02) in the LA and LAA, respectively. A similar 3.0-fold (P<0.01) increase in LAA O$_2^-$ production was observed using a cytochrome C reduction assay. The increases could not be explained by changes in atrial total superoxide dismutase activity. Addition of either apocyanin or oxypurinol reduced LAA O$_2^-$, implying that NADPH and xanthine oxidases both contributed to increased O$_2^-$ production in AF. Enzyme assays of atrial tissue homogenates confirmed increases in LAA NAD(P)H oxidase (P=0.04) and xanthine oxidase (P=0.01) activities. Although there were no changes in expression of the NADPH oxidase subunits, the increase in superoxide production was accompanied by an increase in GTP-loaded Rac1, an activator of the NADPH oxidase.

Conclusions—AF increased O$_2^-$ production in both the LA and LAA. Increased NAD(P)H oxidase and xanthine oxidase activities contributed to the observed increase in LAA O$_2^-$ production. This increase in O$_2^-$ and its reactive metabolites may contribute to the pathological consequences of AF such as thrombosis, inflammation, and tissue remodeling.

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Key Words: atrium ■ superoxide ■ tachycardia

Atrial fibrillation (AF) is the most common sustained arrhythmia in the elderly, affecting >5% of the population >65 years of age. It is considered a progressive disease, increasing in prevalence with age and often advancing from paroxysmal to permanent. AF not only is an independent risk factor for death but also confers significant risk of morbidity from stroke associated with embolization of thrombi developed within the left atrial appendage (LAA). It is estimated that at least two thirds of strokes in patients with AF occur by this mechanism. Despite its prevalence, the cause of AF and localized LAA thrombus formation remains unclear.

Several lines of evidence suggest an association between oxidative stress and AF. Carnes et al have shown that AF induced by rapid pacing in dogs decreases tissue ascorbate levels and increases protein nitration, a biomarker of oxidative and nitrosative stress. Biochemical evidence of oxidation by peroxynitrite and hydroxyl (OH) radicals, both downstream products of O$_2^-$ generation, also has been demonstrated in experimental models.4 CABG, a procedure frequently complicated by AF, is associated with an increase in oxidized glutathione and lipid peroxidation. Angiotensin II (Ang II) stimulates O$_2^-$ production by NAD(P)H oxidases by activation of the type 1 receptor, and inhibition of Ang II production reduces redox stress in the vasculature. Patients with AF are known to have increased levels of ACE and Ang II receptors.6–8 In addition, ACE inhibition has been shown to reduce the incidence of AF in a number of different settings, including after myocardial infarction9 and in the setting of left ventricular systolic dysfunction.10 ACE inhibitor therapy has
been shown to be a negative predictor of AF after CABG, whereas postoperative withdrawal of ACE inhibitors has been associated with the development of AF.\textsuperscript{11}

Recently, we reported that AF caused left atrial (LA) endocardial dysfunction manifested as a 73\% decrease in NO production and a 1.8-fold upregulation of plasminogen activator inhibitor-1 (PAI-1).\textsuperscript{12} One possible explanation for the reduction in NO is increased oxidative degradation by $\text{O}_2^-$. We investigated whether $\text{O}_2^-$ was increased as a result of AF using a pig rapid pacing model of AF.

### Methods

#### Swine Model of AF

Experiments were performed under a protocol approved by the Emory and Atlanta VA Institutional Animal Care and Use committees. The protocol is identical to that previously described.\textsuperscript{13} Pigs (Sus scrofa) weighing 23-44 kg were studied. Using a percutaneous technique, we introduced 2 hemostatic sheaths into the right femoral vein. A 4F quadrilateral catheter was advanced through a sheath and positioned in the apex of the right ventricle for temporary pacing. An ablation catheter (Boston Scientific) was positioned across the AV junction, and radiofrequency energy was applied until complete AV block was produced. Next, the right internal jugular vein was isolated, and a timed, bipolar permanent pacing lead was introduced, advanced under fluoroscopic guidance, and positioned in the apex of the right ventricle. An active fixation bipolar pacing lead was introduced using comparable technique and placed in the right atrial appendage (RAA). In the AF group, the atrial lead was attached to a neurostimulator (Irtel III, Medtronic) positioned subcutaneously in the neck. The device was programmed to a rate of 600 bpm to maintain AF. 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 DDR 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.

After 1 week, animals were reanesthetized. Pacing was withheld, and an ECG was performed to ensure that AF or sinus rhythm was present in the experimental and control groups, respectively. Weights obtained showed no differences among groups, suggesting that pacing had not induced heart failure. Animals were euthanized with sodium pentobarbital intravenously, and the hearts were rapidly excised. The hearts were rinsed and dissected in Krebs-HEPES solution and frozen in liquid nitrogen. Then, the tissue was homogenized in membranes for subsequent enzymatic assays and Western analysis. Sections of the LAA were taken for histological examination.

### Measurements of $\text{O}_2^-$ Using Electron Spin Resonance

#### Western Blotting for NADPH Subunits

Protein samples were prepared from the LAA tissue of control and AF pigs. Frozen tissue pieces were homogenized in Laemml buffer and boiled for 5 minutes. To measure protein concentration, a fraction of each sample was precipitated using 72\% trichloroacetic acid and boiled for 5 minutes. To measure protein concentration, a fraction of each sample was precipitated using 72\% trichloroacetic acid and boiled for 5 minutes.
Rac1 Activation Assay  
Active, GTP-bound Rac1 was precipitated with a PAK-PBD–based assay (Upstate Biotechnology). LAA tissue samples (30 mg) were homogenized in 600 μL of 1× MLB (Upstate Biotechnology) with added protease inhibitors. Samples were pretreated with glutathione agarose, and 500 μL tissue lysates was used for the pull-down assay. A Bradford assay was performed to allow normalization of protein content. PAK-1 PBD agarose (10 μg) was added to each sample and incubated at 4°C for 1 hour. For positive and negative controls, samples were incubated with GTP or GDP, respectively, before PAK-1 PBD agarose was added. Beads were collected, washed 3 times, and resuspended in 40 μL Laemmli buffer. An equal volume of samples (20 μL) was electrophoretically separated at 130 V for 1 hour on a 12.5% SDS polyacrylamide gel, transferred to nitrocellulose membranes, and evaluated for active Rac1 by Western analysis.

Statistical Analysis  
Data are presented as mean±SE. Comparisons between experimental and control animals were performed by use of a Student t test for unpaired data. Multiple means were compared by a 1-way ANOVA and a post-hoc test when significance was indicated. A value of P<0.05 was considered statistically significant.

Results  
After cessation of atrial pacing and at the time of death, all animals in the experimental group were documented to be in AF. Weights did not vary before or after pacing, suggesting that pacing did not cause significant congestive heart failure. Inspection of the atria revealed no gross evidence of thrombus formation. Histological examination by hematoxylin and eosin staining revealed 1 microthrombus in the LAA in 23 animals examined in the experimental group and no thrombi in the control animals.

Intracellular O$_2^-$ Production  
Intracellular O$_2^-$ production from various parts of the heart was examined by ESR using membrane-permeable CMH as a spin probe. The reaction of CMH with O$_2^-$ results in formation of a nitroxide radical that yields a characteristic spectrum (Figure 1). In the control group, O$_2^-$ production was comparable in each of the tissues tested (Figures 1 and 2). The amount of O$_2^-$ was 18.1±2.1 (n=5), 20.2±3.1 (n=5), 27.1±3.9 (n=5), and 23.7±1.8 (n=6) pmol/mg wet weight for the LAA, LA, RAA, and right atrium (RA), respectively. In the experimental animals, 1 week of AF caused 2.7- and 3.0-fold increases in O$_2^-$ production in the LA and LAA over the control measurements, respectively (P<0.02 in each case; Figures 1 and 2). In contrast, AF did not cause significant changes in O$_2^-$ production in the RA or RAA. The amount of O$_2^-$ detected for each segment was 54.6±6.0 (n=4), 53.8±4.9 (n=6), 38.3±4.5 (n=5), and 30.2±6.0 (n=5) pmol/mg wet weight for the LAA, LA, RAA, and RA, respectively (Figure 2).

Extracellular O$_2^-$ Production  
Extracellular O$_2^-$ production from various parts of the heart was examined by SOD-inhibitable cytochrome C reduction.
Superoxide production (normalized to the dry weight of tissue) in control animals was comparable in the various parts of the heart that were assessed (P = 0.70 by 1-way ANOVA; Figure 3). The amount of O$_2^-$ was 21.4±5.4 (n = 21), 13.0±7.3 (n = 6), 12.1±7.3 (n = 6), 15.3±10.0 (n = 6), and 11.5±7.2 (n = 6) pmol/mg dry weight for the LAA, LA, RAA, RA, and coronary artery, respectively.

With 1 week of AF in the experimental group, there was a marked increase in extracellular O$_2^-$ detected in the LAA. In the LAA, AF caused a 3.0-fold increase in O$_2^-$ production over the control measurements (P < 0.01; Figure 3). Compared with control pigs, however, no changes in O$_2^-$ production were observed in the other segments. The amount of O$_2^-$ recorded for each segment was 63.9±8.0 (n = 22), 5.3±5.3 (n = 6), 22.9±11.2 (n = 6), 17.8±11.3 (n = 6), and 11.7±10.4 (n = 7) pmol/mg dry weight for the LAA, LA, RAA, RA, and coronary artery, respectively.

**Determination of the Source of Increased Superoxide During AF**

SODs are a major counter to increased oxidative stress in the vascular endothelium. Therefore, we sought to determine whether the increased O$_2^-$ production observed in AF was the result of reduced SOD activity. Measurements of total SOD activity in the LA, LAA, and aorta showed no change between control and AF animals (Figure 4). Thus, it is unlikely that the increased O$_2^-$ observed in the LA and LAA during AF was due to alterations in SOD activity.

In mammalian cells, the NAD(P)H oxidases are major sources of reactive oxygen species (ROS). Therefore, we examined the role of the NAD(P)H oxidases in the increase in O$_2^-$ production during AF. The NAD(P)H oxidase inhibitor apocynin (100 μg/mL) reduced LAA O$_2^-$ production by 91%, suggesting a role of the NADPH oxidase. To investigate this further, LAA NAD(P)H oxidase activities were compared directly using membrane preparations from AF and control pigs. There was a 4.4-fold increase in NAD(P)H activity in the LAA of pigs with AF (P = 0.02; Figure 5). The

NAD(P)H oxidase activity was 0.4±0.1 versus 1.8±0.5 nmol O$_2^-$ per 1 mg tissue per 1 minute in control versus AF pigs. The LA showed a similar trend toward increased NAD(P)H oxidase activity (P = 0.06).

Because XO is another source of O$_2^-$ that can be activated concomitantly with the NAD(P)H oxidase, we also investigated changes in XO activity caused by AF. Although the overall O$_2^-$ production attributable to this system was lower
Mechanism of Increased NAD(P)H Oxidase Activity During AF

We evaluated whether the increased LAA NADPH oxidase activity with AF was the result of increased expression of the NADPH oxidase subunits or increased oxidase activity. Western blot analysis of Nox1, Nox2 (gp91phox), Nox4, and p22phox showed no change in relative protein amounts caused by AF, suggesting that activation rather than transcriptional regulation was responsible for the increased activity (Figure 6).20–22 The small G protein Rac1 is essential for assembly of the active NAD(P)H oxidase complex.23–25 Therefore, we performed experiments to determine whether AF was associated with an increase in active GTP-bound Rac1. Rac1 activity assays demonstrated that AF increased active Rac1 in the LAA by 6.9-fold (P<0.05) compared with control pigs (Figure 7).

Discussion

Previously, we demonstrated that AF is associated with endocardial dysfunction that may contribute to thrombus formation in the LAA.12 We speculated that the endocardial dysfunction observed during AF might be similar to endothelial dysfunction observed during atherosclerosis. In the case of atherosclerosis, endothelial dysfunction is accompanied by increased production of O$_2^-$. In the present study, we found that AF was also associated with increased intracellular O$_2^-$ production in the LA and LAA and with increased extracellular O$_2^-$ in the LAA. The increased O$_2^-$ production was at least in part the result of increased NAD(P)H oxidase and XO activities. Increased NAD(P)H oxidase activity could be explained by an increase in active Rac1, a required cofactor.

Both ESR and the cytochrome C assay showed similar AF-induced increases in intracellular and extracellular O$_2^-$ production in the LAA. In the LA, however, we found that intracellular O$_2^-$ was increased and extracellular O$_2^-$ production as assessed by the cytochrome C assay was not altered. This difference between the 2 assays might be explained by an increased sensitivity of the ESR technique. It is also possible that there are different enzymatic sources of O$_2^-$ in the LA and the LAA. For example, it has been shown that there are different intracellular distributions of the Nox isoforms in vascular smooth muscle cells that might affect the ability of O$_2^-$ to be released extracellularly.26

Our data indicate that the increase in O$_2^-$ production is greater in the LA and LAA than in the RA or RAA, although a nonsignificant trend toward AF caused an increase in O$_2^-$ in the RA and RAA. Although it is possible that this trend in the RA could reach statistical significance with a larger sample size, it is clear from our data that the increase in O$_2^-$ production is greater in the LA than in the RA. It is uncertain why these differences between the 2 atria are present. Flow profiles, levels of oxygen tension, and mechanical influences, however, clearly vary between the LA and RA. Each of these
factors could influence activation of the NAD(P)H oxidase and XO.

The increased production of ROS may be an important cause of thrombus formation in the LAA in AF, a major risk factor for stroke. In addition to possible oxidative degradation of endocardial NO, O$_2^-$ and ROS derived from O$_2^-$ may affect coagulation cascade components produced by the endocardium. PAI-1 and tissue factor are prothrombotic molecules that are modulated by redox stimuli. Tissue factor activates thrombin, and elevated tissue factor expression is associated with thrombosis in atherosclerosis and sepsis. Tissue factor expression is inhibited tissue factor endothelial NO production. Upregulation of tissue factor may also be facilitated by O$_2^-$ Consistent with these ideas, we have recently reported that AF is associated with a downregulation of LAA NO and increased in PAI-1 and tissue factor.

Our data suggest that the NAD(P)H oxidase is responsible for at least some of the O$_2^-$ produced by the atria in AF. This observation may help explain the link between AF and activation of the renin-angiotensin-aldosterone system, because Ang II is a potent stimulator of the NAD(P)H oxidase. Ang II receptors are upregulated in the LA of humans with AF. ACE inhibition reduces the likelihood of AF in dog models and in patients after myocardial infarction associated with left ventricular dysfunction. Inhibition of renin-angiotensin signaling also reduces the incidence of AF in humans. Although this effect may be due to myriad properties of ACE inhibitors, the NAD(P)H oxidase is potently regulated by Ang II, and a major effect of ACE inhibition or angiotensin receptor antagonism is a reduction in the activity of this enzyme, even in normal animals. Consistent with the importance of Ang II signaling in AF, we have recently shown that cardiac-directed overexpression of ACE results in a phenotype characterized by atrial enlargement and atrial fibrillation.

The mechanism of increased NAD(P)H oxidase activity appeared to be increased enzyme activation. Membrane subunit assembly is required to form an active NAD(P)H oxidase complex. Active, GTP-bound Rac1 is an important determinant of subunit assembly, and the amount of active Rac1 has been shown to correlate with NAD(P)H oxidase–dependent O$_2^-$ production. Although Western blot analysis of Nox subunits showed no changes with AF, AF was associated with a 6.9-fold increase in active Rac1. Possible mechanisms of Rac1 activation include increased Ang II receptor activation, β-receptor activation, or increased cytosolic Ca$^{2+}$. Although not evident, heart failure cannot be excluded as a cause for our findings.

In addition to increased NADPH oxidase activity, we also found increased enzymatic activity of XO in the LAA in AF. The XO inhibitor oxypurinol reduced O$_2^-$ production in these tissues. The observation that both enzyme systems contribute to increased O$_2^-$ production may be the result of interplay between these and other sources of ROS. For example, recent studies have shown that ROS derived from the NADPH oxidase can increase conversion of xanthine dehydrogenase to XO. Likewise, hydrogen peroxide produced from another source such as XO can stimulate the NADPH oxidase.

Together, these observations support a causal relationship between AF and increased ROS production, and localized oxidative stress may explain the dominant role of the LA as a source of AF. It may be that oxidative stress is another mechanism, along with electrical remodeling, that contributes to the vicious cycle of “AF begetting AF.”

In summary, we demonstrate that, in a pacing model, AF causes oxidative stress localized to the LA and LAA that is mediated partially by activation of the NAD(P)H oxidase and XO. The activation of the NAD(P)H oxidase could be explained by increased amounts of the active cofactor Rac1. These findings may help to explain the proclivity of thrombus to form in the LAA and may have implications for the pathogenesis of AF.

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References


CLINICAL PERSPECTIVE

AF is a progressive disease associated with an increased risk of stroke due almost exclusively to emboli from the LAA. Although this problem has been attributed to stasis of blood in the LAA, we recently reported that AF reduces LAA endocardial NO production, which could be explained in part by increased superoxide production. Here, we demonstrate that, in a pacing model, AF increases production of ROS in the LA and LAA and that this increase is mediated by activation of the NAD(P)H oxidase and XO. Activation of the NAD(P)H oxidase was associated with increased amounts of the active cofactor Rac1. Because ROSs are prothrombotic, these findings may help to explain the proclivity for the clot to form in the LAA. Along with or coupled to electrical remodeling, increased radical production also may contribute to the vicious cycle of “AF begetting AF.” There is evidence that therapies known to alter ROS production affect AF. For example, the NAD(P)H oxidase is activated by Ang II. In the TRACE trial, ACE inhibitor use was associated with a lower incidence of AF. In a prospective trial, losartan has been associated with a lower recurrence rate of AF after cardioversion. Results from the present study suggest that therapies designed to diminish ROS production may be useful for reducing the incidence of thromboembolism and AF.
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