Atrial fibrillation (AF) promotes its own maintenance by causing tachycardia-induced electrophysiological and structural remodeling of the atrial myocardium.1,2 One potential link between AF, tachycardia, and atrial remodeling is oxidative stress. Consistent with this hypothesis, rapid atrial pacing has been shown to increase myocardial peroxynitrite formation and lead to a shortening of the atrial effective refractory period (ERP), both of which are reversed by treatment with the antioxidant and peroxynitrite decomposition catalyst ascorbate.3 More recently, administration of other agents with antioxidant and anti-inflammatory properties such as statins has proved similarly effective in preventing ERP shortening and the vulnerability to AF in a dog model of rapid atrial pacing.4

The mechanisms underlying oxidative injury in the fibrillating atrial myocardium remain to be elucidated; however, an increasing body of evidence indicates that formation of the oxygen-derived free radical superoxide by NAD(P)H oxidases plays a critical role in the development of a wide range of cardiovascular diseases,5–7 suggesting that this oxidase system may be an important source of oxidative injury in the fibrillating human atrial myocardium.

In neutrophils, NAD(P)H oxidase consists of a core heterodimer comprising the electron transferring plasma membrane subunits p22phox and gp91phox (or nox2) and 4 cytosolic subunits (p40phox, p47phox, p67phox, and the small G-protein rac1/2), which provide regulatory function.5 In vascular smooth muscle and endothelial cells, gp91phox coexists with other homologues termed nox1 and nox4,8–10 whereas a gp91phox containing NAD(P)H oxidase is the most common isoform in the human ventricular myocardium.7,11

Here, we investigated the source of atrial superoxide production in right atrial appendage (RAA) homogenates and isolated atrial myocytes from patients in sinus rhythm (SR) undergoing cardiac surgery. We then examined whether the presence of AF affected the magnitude or source of superoxide production in RAA homogenates obtained from patients with permanent or paroxysmal AF.

**Materials and Methods**

**Patient Characteristics**

Experiments designed to characterize the source of superoxide production in right atrial tissue (whole tissue homogenates and
changes of the atrial myocardium that are similar to those observed in patients with permanent AF, suggesting that intermittent bursts of AF, particularly if frequent, are sufficient to instigate atrial remodeling in humans.

A preoperative left ventricular ejection fraction (LVEF; %) was obtained in all patients, whereas left atrial size (by thoracoscopic echocardiography) was obtained in 24 (14 in AF and 10 in SR) of the 30 patients included in the matched cohort. Demographic and clinical characteristics of the three groups are shown in Table 1. The study was approved by the local research ethics committee, and all patients gave written informed consent.

### Atrial Myocyte Isolation Protocol

Samples of RAA were digested (40 minutes) in isolation buffer supplemented with type I collagenase (2 mg/mL; Worthington) and protease (0.2 mg/mL; Sigma), 1% BSA, and 50 μmol/L Ca2+, and then transferred into fresh collagenase solution without protease. After isolation, rod-shaped atrial myocytes were enriched (to ~90%) by a two-step centrifugation (3 minutes at 600 rpm) in Kraft–Bruhe medium supplemented with l-glutamate, sodium pyruvate, taurine, creatine, succinic acid, Na3ATP, and β-OH butyrate and used within 3 hours for superoxide measurements.

### Atrial Superoxide Production

Superoxide production in homogenized RAA or isolated atrial myocytes was measured by lucigenin-enhanced chemiluminescence using a single-tube luminometer (Berthold FB12) modified to maintain the sample temperature at 37°C. Briefly, basal chemiluminescence from RAA homogenate or isolated right atrial myocytes was measured in buffer (2 mL) containing lucigenin (5 μmol/L) after reaching equilibrium (~7 minutes). Further measurements were taken after the addition of inhibitors or substrates of specific oxidase systems (Table 2; n=9 to 14 for each comparison). Superoxide scavengers such as polyethylene glycol–superoxide dismutase (PEG-SOD; 650 U/mL) and tiron (10 mmol/L) were also used.

After application of NADPH (100 μmol/L), the chemiluminescence signal in RAA homogenate reached a plateau within 3 minutes; however, in atrial myocytes, the signal continued to increase linearly over time. Hence, in this preparation, measurements do not reflect the maximal NADPH-stimulated superoxide production. Atrial homogenates were separated into soluble (cytosolic) and particulate (membrane-associated) fractions by ultracentrifugation, as described previously.

Because lucigenin (at concentration ≥20 μmol/L) may be subject to redox cycling, we compared basal and NADPH-stimulated superoxide measurements in RAA homogenates by 5 μmol/L lucigenin with those obtained by using the luminol analogue L-012 (100 μmol/L in the presence of ebselen; 50 μmol/L) and the SOD-inhibitable ferricytochrome c reduction, respectively. In both cases, measurements were closely correlated with those obtained in parallel by lucigenin-enhanced chemiluminescence (basal measurements r=0.88, P<0.005, n=8; NADPH-stimulated r=0.99, P<0.0001, n=11), in agreement with previous reports indicating that 5 μmol/L lucigenin is a sensitive and valid probe for assessing superoxide production.
Oxidative Fluorescent Microtopography Using Dihydroethidium

Superoxide production in tissue sections of human RAA was detected using the fluorescent probe DHE. Human RAA were freshly harvested and frozen in OCT compound. Cryosections (30 μm) were incubated in Krebs-HEPES buffer for 30 minutes at 37°C with or without PEG-SOD (1000 U/mL) and with dihydroethidium (DHE; 2 μmol/L; Molecular Probes) for another 5 minutes at 37°C in darkness. Images were obtained using a laser confocal microscope (Bio-Rad; MRC 1024) at identical acquisition settings using an excitation wavelength of 488 nm. Fluorescence was detected at 585 nm.

Immunoblotting

Protein expression of subunits of NAD(P)H oxidase in RAA homogenates was investigated using rabbit polyclonal antibodies directed against cytosolic p47phox and p67phox (07-001 and 07-002; Promega) and a rabbit polyclonal antibody directed against the p22phox subunit (a kind gift from Dr Frans B. Wientjes, University College London, United Kingdom) after separation in 10% SDS-PAGE gels and protein transfer to polyvinylidene difluoride membranes. After incubation in primary antibody, antibody binding was visualized using horseradish peroxidase–conjugated anti-rabbit IgG (Alexis). Fluorescence images were analyzed using ImageJ software (NIH; Bethesda, MD). Densitometric analysis was performed using the ImageJ software (NIH; Bethesda, MD). The absorbance of each lane was normalized to the total protein content in the sample. Data are expressed as the mean ± SEM unless specified otherwise. In all cases, n refers to numbers of patients. Nonparametric statistics was used to evaluate some of the responses to inhibitors or substrates of oxidases and the differences in mRNA expression between AF and SR patients. Other comparisons were made by using ANOVA and Fisher’s protected least significant difference post hoc test. A value of P < 0.05 was considered statistically significant.

Results

Sources of Superoxide Production in RAA

Basal superoxide production was determined by lucigenin (5 μmol/L)-enhanced chemiluminescence in homogenized RAAs and in intact right atrial myocytes (average 80±8 relative light units [RLU]/mg protein; n=39 patients; and 0.031±0.006 RLU/myocyte; n=16 patients, respectively). Specificity for superoxide was demonstrated by near-abolition of chemiluminescence after coincubation with either PEG-SOD (650 U/mL) or tiron (10 mmol/L; Figure 1A). In addition, basal superoxide production was greatly reduced by the inhibitor of flavin-containing oxidases, diphenyleneiodonium (DPI; 100 μmol/L in RAA and 10 μmol/L in atrial myocytes; ≈70%; P<0.005), or by pretreatment with apocynin (≈80%; P<0.005), a specific inhibitor of NAD(P)H oxidases (Figure 1A). A small reduction (10% to 20%) in basal superoxide production was observed in response to inhibition of cyclooxygenase (indomethacin; P=0.047) in isolated myocytes and of mitochondrial complex I (rotenone; P=0.02) in RAA homogenates. In contrast, inhibition of NO
synthase (NOS) with Nω-nitro-arginine methyl ester (L-NAME) tended to increase basal superoxide production (by \( \approx 25\% \) in RAA homogenates; \( P=0.35 \)). Xanthine oxidase inhibition with oxypurinol had no effect on basal superoxide production in both preparations.

NADPH-stimulated superoxide release was markedly inhibited by apocynin and DPI in both preparations, whereas oxypurinol, indomethacin, and L-NAME caused very little change (\( \approx 5\% \) to 10\% reduction; Figure 1B). Rotenone resulted in a small reduction in NADPH-stimulated superoxide release in both preparations (10\% to 20\%; \( P<0.005 \)).

As observed previously in vascular tissue, \(^{23}\) NADPH chemiluminescence was significantly greater (\( \approx 2\)-fold) than the NADH-evoked signal in RAA homogenates and in atrial myocytes. Further experiments showed that NADPH and NADH were the only substrates that elicited a significant increase in superoxide production in both preparations (Figure 2A).

To further characterize myocardial NAD(P)H oxidase activity, we evaluated NADPH-dependent superoxide production in subcellular fractions of RAA and atrial myocyte homogenates (Figure 2B). Subcellular fractionation by ultracentrifugation into soluble (cytosolic) and particulate (membrane) fractions revealed that >95\% of the NADPH-stimulated oxidase activity originated from the particulate fraction in both preparations (\( P<0.005 \) versus cytosolic).

Oxidative fluorescent microtopography using the fluorescent probe DHE showed in situ superoxide production in cryosections of RAAs (Figure 3A), which was significantly attenuated after incubation with PEG-SOD (1000 U/mL).

Together, these data indicate that a membrane-associated NAD(P)H oxidase is the main source of superoxide production in RAA homogenates and right atrial myocytes isolated from patients in SR.
Expression of NAD(P)H Oxidase Subunits in Human RAA Homogenates and Isolated Myocytes

To determine whether NAD(P)H oxidase subunits were expressed in the human atrial myocardium, we used a combined approach using antibodies directed against the p22phox, p47phox, and p67phox subunits of the NAD(P)H oxidase in RAA homogenates and isolated right atrial myocytes and conventional RT-PCR to detect p22phox and gp91phox mRNA in isolated atrial myocytes.

Figure 3B shows that the p22 phox, p47 phox, and p67 phox subunits are detected in RAA homogenate and atrial myocytes. p22phox, p47phox, and p67phox appeared as single bands, which were aligned to their respective positive control bands (human HL-60 or EB-1 neutrophils lysate). C, RT-PCR for p22phox and gp91phox subunits in atrial myocytes. M represents mass ladder; + (colored lanes), positive controls. Negative controls (red lanes) were obtained by omission of reverse transcriptase. Test lanes are marked in yellow. D, Immunolocalization of the p47phox and p67phox subunits of the NAD(P)H oxidase in myocytes isolated from RAAs. Negative control was obtained by omission of the primary antibody.

Figure 3. DHE staining and NAD(P)H oxidase subunit expression in RAA and human atrial myocytes from patients in SR. A, 2 μmol/L DHE staining for superoxide in basal (negative control [NC]) and PEG-SOD–treated cryosections of human RAA. B, Immunoblotting of the p22phox, p47phox, and p67phox subunits of NAD(P)H oxidase in RAA homogenates and isolated right atrial myocytes. + Positive controls (human HL-60 or EB-1 neutrophils lysate). C, RT-PCR for p22phox and gp91phox subunits in atrial myocytes. M represents mass ladder; + (colored lanes), positive controls. Negative controls (red lanes) were obtained by omission of reverse transcriptase. Test lanes are marked in yellow. D, Immunolocalization of the p47phox and p67phox subunits of the NAD(P)H oxidase in myocytes isolated from RAAs. Negative control was obtained by omission of the primary antibody.

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p47phox or p67phox immunolocalization in isolated human atrial myocytes showed a diffuse pattern consistent with cytosolic and membrane-bound localization of these NAD(P)H oxidase subunits (Figure 3D). Omission of the primary antibody (negative control) resulted in no staining in 17 of 17 cells from 3 patients. Corresponding phase contrast images of the same cells are shown in the bottom panel of Figure 3D.

Atrial NAD(P)H Oxidase Activity Is Increased in Patients With AF

NADPH-stimulated superoxide production was significantly higher in RAA homogenates derived from AF patients compared with patients in SR (Figure 4A; P=0.02), who were matched for age, LVEF (46±12% in SR and 50±13% in AF; P=0.40), treatment, and known risk factors (Table 1). Basal superoxide production also tended to be higher in the AF group, but this difference did not reach statistical significance (P=0.15; Figure 4A). LVEF was not different between AF and SR patients (Table 1). As expected, atrial size was significantly larger in the AF group (4.05±0.16 cm in SR patients; n=10 versus 5.01±0.17 cm in AF patients; n=14; P=0.0006); however, there were no differences in LVEF (P=0.97), atrial size (P=0.38), or superoxide production (P=0.32) between patients with permanent or paroxysmal AF. Similarly, we did not find a significant correlation between atrial size (range 3.3 to 6 cm) and basal or NADPH-stimulated superoxide production (r²=0.03 for both), suggesting that the latter is unlikely to be a consequence of atrial hypertrophy and remodeling in AF.

As observed in patients in SR, basal superoxide production in RAA homogenates from AF patients was inhibited by apocynin (≈81%; P=0.005) and rotenone (≈40%; P=0.02; Figure 4B; P=0.55 and 0.33, respectively, for comparison with the apocynin- and rotenone-induced inhibition in SR
patients). Interestingly, in fibrillating RAA, pretreatment with 1-NAME (but not with L-NAME; 1 mmol/L; n=3; data not shown) significantly reduced basal superoxide production by \( \approx 40\% \) (\( P=0.01 \) versus control and \( P=0.002 \) versus SR), indicating that dysfunctional NOS may become a significant source of basal superoxide production in fibrillating atria. Similar results were obtained by using \( N^{\text{G}}\)-monomethyl-L-arginine (L-NMMA; 1 mmol/L; n=3; data not shown).

Pretreatment with apocynin significantly decreased NADPH-stimulated superoxide release (\( \approx 90\% \); \( P=0.005 \)), and an \( \approx 20\% \) reduction in NADPH-stimulated superoxide release was also observed in response to 1-NAME (\( P=0.008 \) versus control and \( P=0.07 \) versus SR; Figure 4B). Rotenone, oxypurinol, and antimycin-A (10 \( \mu \)mol/L) had no significant effect.

Quantification of \( p22^{\text{phox}} \) and \( gp91^{\text{phox}} \) mRNA in RAA from patients with AF and matched controls in SR using real-time RT-PCR showed no difference in the expression of these subunits between groups (n=15 patients per group, arbitrary copy numbers; \( p22^{\text{phox}} \) 328.5\( \pm \)71.4 in AF versus 280.1\( \pm \)37.5 in SR; \( gp91^{\text{phox}} \) 139.9\( \pm \)51 in AF versus 138.6\( \pm \)27.1 in SR; \( P=\text{NS} \); Figure 5).

**Discussion**

The main findings of this study are as follows: (1) a membrane-bound \( gp91^{\text{phox}}/\text{nox2} \) containing NAD(P)H oxidase is the main source of superoxide production in human atrial myocytes; (2) NAD(P)H-dependent superoxide production from RAA homogenates is increased in patients with a history of AF in the absence of changes in the mRNA expression of the \( p22^{\text{phox}} \) and \( gp91^{\text{phox}}/\text{nox2} \) subunits; and (3) in atrial tissue from patients with AF, NOS inhibition resulted in a significant reduction in lucigenin-enhanced chemiluminescence, suggesting that NOS may be “uncoupled” and contribute to superoxide production in fibrillating atria.

These findings suggest that a myocardial NAD(P)H oxidase, and, to a lesser extent, NOS and mitochondrial oxidases may play an important role in the atrial oxidative injury and electrophysiological remodeling observed in patients with AF.

**Characterization of the Human Atrial NAD(P)H Oxidase**

Our findings indicate that a membrane-bound \( gp91^{\text{phox}} \) NAD(P)H oxidase is present in human right atrial myocytes, where it constitutes the main source of superoxide production. This is evidenced by the observation that RAA superoxide production is significantly increased by the addition of NADPH or NADH but not by application of substrates of other oxidase systems. Similarly, basal and NADPH-stimulated superoxide production (the latter resulting almost exclusively from the particulate fraction) were nearly abolished by apocynin and DPI but not by inhibitors of other oxidases. Pretreatment of RAA homogenates with rotenone and antimycin-A revealed a trend toward a reduction in basal superoxide production, suggesting that the contribution of mitochondrial oxidases to basal superoxide production in the atria is reduced in patients with AF.
human atrial myocardium is small. We also provide evidence of atrial myocyte-specific expression of the NAD(P)H oxidase subunits p22^phox, p47^phox, and p67^phox at the protein level and of p22^phox and gp91^phox/nox2 (but not nox1 or nox4) at the messenger level.

Together, our findings indicate that human atrial myocytes have the capability of producing superoxide through a membrane-bound gp91^phox/nox2 containing NAD(P)H oxidase.

**Myocardial Superoxide Production, Oxidative Stress, and AF**

Increased atrial superoxide production may have important implications in the ionic remodeling process, which promotes AF maintenance. Enhanced myocardial superoxide production can decrease NO bioavailability by scavenging NO (to form the potent oxidant peroxynitrite) and by “uncoupling” NOS activity,25,26 which in turn may lead to an increase in myocardial oxygen consumption,27 β-adrenergic responsiveness,28,29 and thrombogenic risk.30

Carnes et al31 reported an increase in atrial 3-nitrotyrosine content (a marker of peroxynitrite formation in vivo) in a canine model of rapid atrial pacing that was associated with an abbreviation of the atrial ERP. Interestingly, administration of the antioxidant ascorbate attenuated atrial peroxynitrite formation and electrophysiological remodeling in these animals. Similarly, statins have been shown recently to reduce angiotensin II–stimulated (but not basal) NAD(P)H oxidase activity in human RAA31 and to attenuate ERP reduction and AF inducibility in dogs exposed to rapid atrial pacing.31 Together, these findings suggest the presence of a causal relationship between pro-oxidative cellular redox state, atrial ionic remodeling, and AF.

Our findings clearly show that NAD(P)H oxidase is the main source of atrial superoxide production in AF and in SR; however, they also indicate that the contribution of NO to myocardial superoxide production is significantly increased in the fibrillating atrial myocardium. This is evidenced by a reduction in basal- and NADPH-stimulated superoxide production after pretreatment with L-NAME or L-NMMA in RAA homogenates from AF patients. These data differ significantly from those obtained in patients in SR, in whom L-NMMA tended to increase lucigenin chemiluminescence.

It is now well established that NOSs can release superoxide when deprived of their critical cofactor tetrahydrobiopterin (BH4) or of their substrate L-arginine.32 Under these conditions (referred to as “NOS uncoupling”), electron flow results in a reduction of molecular oxygen at the prosthetic heme site of the enzyme rather than in NO synthesis. In the presence of increased oxidative stress, oxidation of BH4 can uncouple NOS to generate reactive oxygen species.26,33 The notion that this may occur in the fibrillating atrial myocardium is supported by the recent findings of Cai et al,30 who showed that eNOS expression in the left atrial appendage in a porcine model of rapid pacing-induced AF did not differ from control animals despite a documented reduction in NO formation at this site.

We observed that the capacity of NAD(P)H oxidase to generate superoxide was significantly higher in patients with AF compared with matched patients in SR. Enhanced NAD(P)H oxidase activity can either be attributable to increased expression or post-translational modifications of the oxidase subunits. Real-time quantitative RT-PCR revealed no difference in p22^phox or gp91^phox mRNA between AF and SR groups, suggesting that the increase in NAD(P)H oxidase activity observed in AF may be attributable to the latter mechanism. Indeed, a key feature of cardiovascular NAD(P)H oxidase is its responsiveness to hormones, hemodynamic forces, and local metabolic changes.5 In particular, vascular NAD(P)H oxidase activity is known to be greatly increased by angiotensin II via phosphorylation of p47^phox.34 In agreement with these findings, we found that angiotensin II potently stimulates atrial NAD(P)H oxidase activity (Kim et al, unpublished observations, 2004) which, like its vascular counterpart, is markedly inhibited by chelerythrine, indicating that these effects are at least in part protein kinase C dependent. Together, these findings suggest that the activation of the renin-angiotensin system may be an important underlying mechanism for the increased NAD(P)H oxidase activity and ensuing atrial oxidative stress observed in human AF.

**Limitations**

The findings of our study should be interpreted in the context of their limitations. Because regional differences in endocardial NO production have been reported in a porcine model of AF induced by rapid right atrial pacing,30 caution should be exerted in extrapolating our findings to the whole atrial myocardium. However, in humans, AF has been shown to cause similar changes in size, function,35 and ion channel protein expression14 in both atria, suggesting that remodeling may be a diffuse phenomenon.

To our knowledge, our study provides the first evidence of NOS uncoupling in the human myocardium; however, detailed investigation of the mechanisms responsible for this phenomenon was hampered by limited tissue availability because the incidence of AF in patients undergoing first-time elective coronary revascularization was <10%. Because all our SR controls patients underwent coronary artery bypass surgery, we elected to recruit AF patients from a similar cohort to match the two groups for risk factors (eg, age, atherosclerosis, and diabetes mellitus) and treatment agents (eg, statins, angiotensin-converting enzyme inhibitors, or angiotensin II receptor blockers) that are known to affect myocardial/endothelial superoxide production.9,31

**Conclusions**

Increasing evidence indicates that NAD(P)H oxidase may play an important role in the myocardial response to stress or injury. Our findings indicate that the primary source of superoxide production in the human atrial myocardium is an NAD(P)H oxidase in patients in SR and in those with AF. However, in the fibrillating myocardium, NOS contributes significantly to basal- and NADPH-stimulated superoxide release, suggesting that increased oxidative stress in this condition may lead to NOS uncoupling with potentially important implications on myocardial function and thrombogenesis.
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A Myocardial Nox2 Containing NAD(P)H Oxidase Contributes to Oxidative Stress in Human Atrial Fibrillation
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