A Myocardial Nox2 Containing NAD(P)H Oxidase Contributes to Oxidative Stress in Human Atrial Fibrillation

Young M. Kim, Tomasz J. Guzik, Yin Hua Zhang, Mei Hua Zhang, Hassan Kattach, Chandi Ratnatunga, Ravi Pillai, Keith M. Channon, Barbara Casadei

Abstract—Human atrial fibrillation (AF) has been associated with increased atrial oxidative stress. In animal models, inhibition of reactive oxygen species prevents atrial remodeling induced by rapid pacing, suggesting that oxidative stress may play an important role in the pathophysiology of AF. NAD(P)H oxidase is a major source of superoxide in the cardiovascular system; however, whether this enzyme contributes to atrial oxidative stress in AF remains to be elucidated. We investigated the sources of superoxide production (using inhibitors and substrates of a range of oxidases, RT-PCR, immunofluorescence, and immunoblotting) in tissue homogenates and isolated atrial myocytes from the right atrial appendage (RAA) of patients undergoing cardiac surgery (n=54 in sinus rhythm [SR] and 15 in AF). A membrane-bound gp91phox containing NAD(P)H oxidase in atrial myocytes was the main source of atrial superoxide production in SR and in AF. NADPH-stimulated superoxide release from RAA homogenates was significantly increased in patients with AF in the absence of changes in mRNA expression of the p22phox and gp91phox subunits of the NAD(P)H oxidase. In contrast with findings in SR patients, NO synthases (NOSs) contributed significantly to atrial superoxide production in fibrillating atria, suggesting that increased oxidative stress in AF may lead to NOS “uncoupling.” These findings indicate that a myocardial NAD(P)H oxidase and, to a lesser extent, dysfunctional NOS contribute significantly to superoxide production in the fibrillating human atrial myocardium and may play an important role in the atrial oxidative injury and electrophysiological remodeling observed in patients with AF. (Circ Res. 2005;97:0-0.)

Key Words: atrial fibrillation • humans • nitric oxide synthase • NAD(P)H oxidase • superoxide

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 in 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...
isolated intact atrial myocytes) were performed in RAAs obtained from 39 patients in SR who underwent first-time coronary artery bypass surgery with or without mitral/aortic valve replacement.

Because lucigenin (at concentration 0.20 μmol/L) may be subject to redox cycling, we compared basal and NADPH-stimulated superoxide measurements in RAAs homogenates by 5

TABLE 2. Inhibitors and Substrates of Specific Oxidase Systems

<table>
<thead>
<tr>
<th>Oxidase System</th>
<th>Inhibitor</th>
<th>Substrate</th>
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<tr>
<td>Mitochondrial oxidative complex III</td>
<td>Antimycin-A (10 μmol/L)</td>
<td>Succinate (5 mmol/L)</td>
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<tr>
<td>Mitochondrial oxidative complex I</td>
<td>Rotenone (100 μmol/L)</td>
<td>NADPH or NADH (100 μmol/L)</td>
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<tr>
<td>NAD(P)H oxidases</td>
<td>Apocynin (100 μmol/L)</td>
<td>NADPH or NADH (100 μmol/L)</td>
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<tr>
<td>Flavin-containing oxidases</td>
<td>DPI (10 or 100 μmol/L)</td>
<td>NADPH or NADH (100 μmol/L)</td>
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<tr>
<td>Cyclooxygenase</td>
<td>Indomethacin (100 μmol/L)</td>
<td>Arachidonic acid (100 μmol/L)</td>
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<tr>
<td>Xanthine oxidase</td>
<td>Oxypurinol (100 μmol/L)</td>
<td>Xanthine (1 mmol/L)</td>
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<tr>
<td>NOSs</td>
<td>L-NAME or L-NMMA (1 mmol/L)</td>
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Oxidative Fluorescent Microtopography Using Dihydroethidium

Superoxide production in tissue sections of human RAAs was detected using the fluorescent probe DHE. Human RAAs were freshly isolated 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.

Immunolocalization

Protein expression of subunits of NAD(P)H oxidase in RAA homogenates was investigated using rabbit polyclonal antibodies directed against cytosolic p47phox and p67phox subunits (07-001 and 07-002; Upstate) 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 obtained with the primary antibody, antibody binding was visualized using a laser confocal microscope (Bio-Rad; MRC 1024) at identical acquisition settings 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

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Real-Time RT-PCR

Conventional RT-PCR was initially used to evaluate the presence of gp91phox/nox2, its homologues nox1 and nox4, and p22phox in RAA homogenates of patients in SR. Optimal PCR conditions were 100 nmol/L primers for p22phox, gp91phox, nox1, and nox4 (Table 3), 2.5 mmol/L MgCl₂ for p22phox, gp91phox, and nox1, and 4.0 mmol/L MgCl₂ for nox4. Annealing temperatures were 58°C for all primers except nox4 (68°C) and extension at 72°C. Total RNA extracted from human THP-1 cells (monocytic cell line) served as positive controls for the detection of p22phox and gp91phox, and RNA derived from human EA (endothelial cell line) cells provided positive control for the presence of nox1 and nox4. PCR amplification for p22phox, gp91phox, nox1, and nox4 cDNA generated 341-, 225-, 377-, and 516-bp fragments, respectively, and were resolved on 2% agarose gel.

Quantitative real time RT-PCR (Rotor-Gene 3000; Corbett Research) was used to compare the expression level of the primary transcripts for p22phox and gp91phox/nox2 subunits of NAD(P)H oxidase in patients with AF and their matched controls in SR. Total RNA was extracted from RAA using a double Trizol extraction procedure, with the resulting RNA pellet treated with amplification grade DNase (Invitrogen). Total RNA was then quantified using RiboGreen, adjusted to 25 ng/μL, and then subjected to one-step reverse transcription and PCR amplification (Table 2) using QuantiTect SYBR Green (Qiagen) in the following conditions: 2.5-pmol/L primers for gp91phox and p22phox, 2.5 mmol/L MgCl₂, annealing at 60°C, extension at 72°C. Arbitrary copy numbers were analyzed using Rotor-Gene v.5 software (Corbett Research) from standard curves generated from serial dilutions of total RNA extracted from RAAs for human p22phox and gp91phox templates.

Statistical Analysis

Data are expressed as 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 Homogenates and Isolated Myocytes From Patients in SR

Basal superoxide production was determined by lucigenin (5 μmol/L)-enhanced chemiluminescence in homogenized RAAs and in intact right atrial myocytes (average 80±8 RU/mg protein; n=39 patients; and 0.03±0.006 RU/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, diphenyleioumomium (DPI; 100 μmol/L in RAA and 10 μmol/L in atrial myocytes; ≈70%; P<0.005), or by pretreatment with apocyclin (≈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 (rottenone; P=0.02) in RAA homogenates. In contrast, inhibition of NO

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<th>TABLE 3. PCR Primers</th>
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<tr>
<td><strong>Human Gene</strong></td>
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<tr>
<td>gp91phox (nox2)</td>
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<td>p22phox</td>
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F indicates forward; R, reverse.
synthase (NOS) with N-methyl-L-arginine (L-NAME) tended to increase basal superoxide production (by ca. 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 (ca. 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 (ca. 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 atrial myocytes and conventional RT-PCR to detect p22phox and gp91phox mRNA in isolated atrial myocytes.

Figure 3B shows that the p22phox, p47phox, and p67phox 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 neutrophil lysate). Conventional RT-PCR confirmed the presence of p22phox and gp91phox mRNA in purified human atrial myocytes and conventional RT-PCR to detect p22phox and gp91phox mRNA in isolated atrial myocytes.

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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, fibrillating RAA pretreatment with L-NAME (but not with D-NAME; 1 mmol/L; n=11005; data not shown) significantly reduced basal superoxide production by 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 L-nitro-arginine (L-NMMA; 1 mmol/L; n=3; data not shown).

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

Quantification of p22phox and gp91phox 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; p22phox 328.5 ± 71.4 in AF versus 280.1 ± 37.5 in SR; gp91phox 139.9 ± 51 in AF versus 138.6 ± 27.1 in SR; P=NS; Figure 5).

**Discussion**

The main findings of this study are as follows: (1) a membrane-bound gp91phox/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 p22phox and gp91phox/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 gp91phox 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.

**Figure 4.** A, Basal- and NADPH-stimulated superoxide production in RAA homogenates from patients with AF and matched controls in SR (n=15 per group; P<0.05). B, Sources of superoxide production in RAA homogenates as described in Figure 1. Bars show means±SEM; *P<0.05.

**Figure 5.** Quantitative real time RT-PCR of p22phox and gp91phox mRNA in RAA homogenates. Total RNA was normalized to 25 ng/μL using fluorometric quantification. Arbitrary copy units were generated from RT-PCR of 0.5 ng total RNA for p22phox and gp91phox genes in NSR and AF (n=15 each group; P=NS).
superoxide production, suggesting that the contribution of mitochondrial oxidases to basal superoxide production in the human atrial myocardium is small. We also provide evidence of atrial myocyte-specific expression of the NAD(P)H oxidase subunits p22phox, p47phox, and p67phox at the protein level and of p22phox and gp91phox/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 gp91phox/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, which in turn may lead to an increase in myocardial oxygen consumption, β-adrenergic responsiveness, and thrombogenic risk.

Carnes et al 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 RAA and to attenuate ERP reduction and AF inducibility in dogs exposed to rapid atrial pacing. 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 NOS 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. 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. The notion that this may occur in the fibrillating atrial myocardium is supported by the recent findings of Cai et al, 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 p22phox or gp91phox 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. In particular, vascular NAD(P)H oxidase activity is known to be greatly increased by angiotensin II via phosphorylation of p47phox. In agreement with these findings, we found that angiotensin II potently stimulates atrial NAD(P)H oxidase activity (Kim et al, unpublished observations) 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, 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, and ion channel protein expression 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 I receptor blockers) that are known to affect myocardial/endothelial superoxide production.

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 eNOS uncoupling with potentially...
important implications on myocardial function and thrombogenesis.

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