Atrial Sources of Reactive Oxygen Species Vary With the Duration and Substrate of Atrial Fibrillation
Implications for the Antiarrhythmic Effect of Statins

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Background—An altered nitric oxide–redox balance has been implicated in the pathogenesis of atrial fibrillation (AF). Statins inhibit NOX2-NADPH oxidases and prevent postoperative AF but are less effective in AF secondary prevention; the mechanisms underlying these findings are poorly understood.

Methods and Results—By using goat models of pacing-induced AF or of atrial structural remodeling secondary to atrioventricular block and right atrial samples from 130 patients undergoing cardiac surgery, we found that the mechanisms responsible for the NO-redox imbalance differ between atria and with the duration and substrate of AF. Rac1 and NADPH oxidase activity and the protein level of NOX2 and p22phox were significantly increased in the left atrium of goats after 2 weeks of AF and in patients who developed postoperative AF in the absence of differences in leukocytes infiltration. Conversely, in the presence of longstanding AF or atrioventricular block, uncoupled nitric oxide synthase activity (secondary to reduced BH4 content and/or increased arginase activity) and mitochondrial oxidases accounted for the bialtral increase in reactive oxygen species. Atoxavastatin caused a mevalonate-reversible inhibition of Rac1 and NOX2-NADPH oxidase activity in right atrial samples from patients who developed postoperative AF, but it did not affect reactive oxygen species, nitric oxide synthase uncoupling, or BH4 in patients with permanent AF.

Conclusions—Upregulation of atrial NADPH oxidases is an early but transient event in the natural history of AF. Changes in the sources of reactive oxygen species with atrial remodeling may explain why statins are effective in the primary prevention of AF but not in its management. (Circulation. 2011;124:1107-1117.)

Key Words: atrial fibrillation ■ free radicals ■ nitric oxide synthase ■ oxidative stress ■ statins

Atrial fibrillation (AF), the most common sustained cardiac arrhythmia, is associated with significantly increased morbidity and mortality.1 A striking feature of AF is its ability to sustain itself by inducing a number of electric and structural changes in the atrial myocardium, which in turn promote AF maintenance and increase vulnerability to relapse.2 Although the process of AF-induced atrial remodeling and its role in begetting AF have been described in great detail in animal models of atrial tachyarrhythmia and in human AF,2 the underlying mechanisms that result in these electric and structural changes remain unclear. Emerging data indicate that an altered nitric oxide (NO)–redox balance in the atrial myocardium may be implicated in the pathogenesis of AF and AF-induced atrial remodeling. In particular, in human and experimental atrial tachyarrhythmia, increased atrial production of reactive oxygen species (ROS) and reduced NO bioavailability are associated with atrial remodeling and increased vulnerability to AF.3–7 Both of which can be prevented by agents that decrease myocardial oxidative stress and inflammation.7–9 In humans, NOX2-containing NADPH oxidases are the main source of ROS production in both right atrial (RA) homogenates and isolated myocytes,6 and RA NADPH-stimulated superoxide production is independently associated with an increased risk of developing AF after cardiac surgery.10 Statins prevent AF-induced early electric remodeling in dogs8 and reduce the occurrence of postoperative AF in patients undergoing cardiac surgery11 but are less effective in the secondary prevention of AF12–14 or in the presence of significant atrial structural remodeling (eg, in heart failure15). By inhibiting HMG CoA reductase, statins prevent the isoprenylation of Rac1 and reduce ROS formation by NADPH oxidases in cardiac tissue.16,17 In human endo-
thelial cells, statins have also been shown increase endothelial NO synthase (eNOS) expression and the synthesis of the NOS cofactor tetrahydrobiopterin (BH₄). If these ancillary effects of HMG CoA reductase inhibition are relevant to the prevention of AF, changes in atrial ROS production or its enzymatic sources with the duration and substrate of AF may affect the antiarrhythmic efficacy of statins.

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To test this hypothesis, we investigated time-dependent changes in the expression and activity of atrial ROS-generating systems in the RA and left atrial (LA) myocardi-um of goats after 14 days (ie, at a stage when electrophysiological remodeling is completed but structural changes are minimal) and 6 months (when atrial structural remodeling is fully established) of pacing-induced AF and in their controls in sinus rhythm (SR) with or without atrial structural remodeling secondary to 4 weeks of complete atrioventricular block (AVB) induced by atrioventricular node ablation. The relevance of these findings to postoperative and permanent AF in humans was assessed in samples of the RA appendage from 26 patients with permanent AF (first documented 1.5 to 22 years before surgery; mean 6.5 ± 1 years) and 104 patients in SR who had no history of AF and were not taking any antiarrhythmic medication other than β-blockers. Of these, 72 remained in SR after surgery and 32 developed postoperative AF (within a week from surgery).

The study was approved by the local Research Ethics Committee, and all patients gave written informed consent. All materials were purchased from Sigma (St. Louis, MO) unless specified.

Superoxide production from atrial samples was measured by use of both lucigenin (5 μmol/L)-enhanced chemiluminescence and 2-hydroxyethidium (2-OHE) detection by high-performance liquid chromatography. To elucidate the contribution of specific oxidases to basal atrial superoxide production, samples were pretreated with the following inhibitors: apocynin or TPCK (100 μmol/L), N-nitro-l-arginine methyl ester (L-NAME) caused a significant reduction in superoxide release in 6M-AF and AVB (E and F), whereas xanthine oxidase inhibition with oxypurinol (Oxy) did not affect atrial superoxide production in any of the groups. Data are expressed as mean ± SEM. *P < 0.05 vs SR; †P < 0.05 vs 2 weeks of AF; ‡P < 0.05 vs control; 1-way ANOVA (n = 10 to 19).

Methods

Forty-eight female goats (weight, 45 to 61 kg) were anesthetized and instrumented, as described in detail previously. After recovery from surgery, AF was induced by repetitive burst pacing. Fifteen goats were euthanized 2 weeks after the induction of AF, and 10 goats were euthanized after 6 months of AF. Goats in SR (n = 23) with (n = 9) or without (n = 14) atrial structural remodeling caused by AVB were used as controls for 6-month and 2-week AF, respectively. The study was performed according to the European directive on laboratory animals (86/609/EEC) and was approved by the local ethics committee.

Samples of the RA appendage were obtained from 26 patients with permanent AF (first documented 1.5 to 22 years before surgery; mean 6.5 ± 1 years) and 104 patients in SR who had no history of AF and were not taking any antiarrhythmic medication other than β-blockers. Of these, 72 remained in SR after surgery and 32 developed postoperative AF (within a week from surgery).
the ratio of GTP-Rac1 to total Rac1, was evaluated by an affinity precipitation assay with PAK1–PBD–conjugated glutathione agarose beads, according to the manufacturer’s instructions (Millipore, Temecula, CA). Immunostaining for CD18 (R&D Systems, Minneapolis, MN) and α-actinin was performed in human RA samples and goat LA tissue blocks embedded in optimal cutting temperature media. An expanded Methods section is included in the online-only Data Supplement.

**Results**

**Time-Dependent Changes in the Sources of Atrial Superoxide Production in a Goat Model of Atrial Fibrillation or Atrioventricular Block–Induced Atrial Structural Remodeling**

Short-term (2 weeks) AF was associated with a significant increase in basal and NADPH-stimulated superoxide production (measured both by lucigenin-enhanced chemiluminescence and 2-OHE detection by high-performance liquid chromatography; Figure 1A through 1C) in the LA myocardium, which was significantly reduced by NADPH oxidase inhibition with apocynin (Figure 1D) or by inhibition of flavin-containing enzymes with DPI (by 71±2.6%; P<0.01). In contrast, a bivariate increase in basal, but not NADPH-stimulated, superoxide production was observed after 6 months of AF and in the presence of AVB (Figure 1A through 1C). Under these conditions, neither apocynin (Figure 1E and 1F) nor TPCK (an alternative inhibitor of NADPH oxidase; data not shown) had any effect on atrial superoxide produc-

**Statistical Analysis**

All values are expressed as mean±SEM. The Student unpaired t test was used in 2-group comparisons of normally distributed data, whereas the Mann-Whitney nonparametric test or Kruskal-Wallis 1-way ANOVA (for multiple-group comparisons) was used when the normality assumption was not met. Multiple groups of normally distributed data of similar variance were compared by 1-way ANOVA. For these comparisons, Bonferroni-corrected P values were given, and a threshold of P<0.05 was used. The χ² test was used to compare sex distribution, smoking status, and medical history between groups; the Fisher exact test was used to compare surgical procedures. A value of P<0.05 was considered statistically significant.
tion, whereas preincubation with the mitochondrial complex 1 inhibitor rotenone reduced atrial superoxide to the level observed in SR (Figure 1E and 1F). Inhibition of xanthine oxidase with oxypurinol did not affect superoxide production in any of the groups (Figure 1D through 1F). In agreement with these findings, the protein level of the NOX2 and p22phox subunits of NADPH oxidase in the LA was increased after 2 weeks of AF but was unchanged after 6 months of AF or in the presence of AVB (Figure 2A). Leukocytes are a major source of NOX2-NADPH oxidases, and in these cells, superoxide generation typically involves the activation of \( \beta_2 \) integrin (CD18).\(^\text{25} \) To investigate whether the increased NOX2 expression and activity in the LA myocardium of goats after 2 weeks of AF were due to myocardial leukocytes infiltration, we compared LA CD18 staining in goats in SR, 2-week AF, and 6-month AF. As shown in Figure 3A, positivity for CD18 staining was found in all samples, but it did not differ between groups.

After 6 months of AF and in the presence of AVB, pretreatment with L-NAME resulted in a reduction in superoxide release in the RA myocardium (Figure 1E and 1F), suggesting the presence of uncoupling of NOS activity (a phenomenon whereby the catalytic electron flow within the enzyme is uncoupled from NO synthesis and diverted to molecular oxygen to yield superoxide\(^\text{26} \)). Reduced bioavailability of the NOS cofactor BH\(_4\) or the substrate l-arginine\(^\text{27} \) or altered eNOS phosphorylation\(^\text{28} \) may lead to NOS uncoupling. As shown in Figure 2B and 2C, BH\(_4\) content and the

Figure 3. Staining for the leukocyte marker CD18 (shown in red) did not differ between groups in both goat left atrial (LA; A) and human right atrial (RA; B) tissue. \( \alpha \)-Actinin is shown in green. All data are expressed as mean±SEM. SR indicates sinus rhythm; 2W-AF, 2 weeks of atrial fibrillation; 6M-AF, 6 months of AF; PostAF, postoperative AF; and Pe-AF, permanent AF.
presence of longstanding AF or AVB, mitochondrial oxidases NADPH oxidase expression and activity, whereas in the production after 2 weeks of AF is driven by an increase in LA expression was modestly reduced, but the eNOS Ser-1177 activity was increased (Figure 2D). Total eNOS protein terins did not change significantly (Figure 2B), but arginase increased (Figure 2D). In the presence of AVB, RA biop-

ratio between BH4 and its oxidized products (BH2 and biopterin) were significantly decreased in the RA myocardium of goats (which compete with NOS for L-arginine) was significantly increased (Figure 4D), resulting in a reduction in the ratio of BH4 to BH2

SR indicates sinus rhythm before and after surgery; AF, atrial fibrillation; CABG, coronary artery bypass surgery; AVR, aortic valve replacement; MVR, mitral valve replacement; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; LVEF, left ventricular ejection fraction; ACEI, angiotensin-converting enzyme inhibitor; and ARB, angiotensin II receptor blocker. *P<0.05.

The Sources of Atrial Superoxide Differ Between Patients Who Develop Atrial Fibrillation After Cardiac Surgery and Those With Permanent Atrial Fibrillation

We have previously shown that NOX2-containing NADPH oxidases are the main source of superoxide production in the human RA myocardium6 and that atrial NADPH-stimulated superoxide production is independently associated with a higher risk of developing AF after cardiac surgery.19 Here, we compared the production and sources of RA superoxide in patients who developed AF after cardiac surgery and those with permanent AF. Demographic and clinical characteristics of these patients are shown in the Table.

Patients who developed postoperative AF showed a DPI- and apocynin-reversible increase in RA basal superoxide release (Figure 4A), which was associated with an increased tissue level of the NOX2 and p22phox subunits of NADPH oxidase (Figure 4B). As shown in Figure 3B, RA CD18 staining was not increased in patients who developed AF after surgery (compared with patients who remained in SR or those who were in permanent AF), again indicating that differences in neutrophil infiltration did not account for the increase in NOX2 activity in the postoperative AF group. Inhibition of NOS with L-NAME did not affect RA superoxide production (Figure 4A), indicating that atrial NOS activity remained “coupled” in patients who developed postoperative AF despite the increase in NADPH oxidase activity. In line with this finding, RA BH4 content was unaltered (Figure 4C), although the oxidized products of BH4 (BH2 and biopterin) were increased (Figure 4D), resulting in a reduction in the ratio of BH4 to BH2+biopterin (Figure 4E).

Right atrial samples from patients with permanent AF showed a significantly higher basal superoxide production (as assessed by lucigenin-enhanced chemiluminescence [Figure 5A] and 2-OHE detection assay [Figure 5B]) that was not inhibited by apocynin or oxypurinol. As observed in the goat model of long-term AF, the protein level and gene expression of the NOX2 subunit of NADPH oxidases in the human RA myocardium were unaffected by permanent AF (Figure 5C). Because apocynin is thought to inhibit predominantly NOX2-containing NADPH oxidases,20 we investigated whether the protein and mRNA expression of NOX homologs (which do not require membrane translocation of cytosolic subunits for their activation and therefore might not be inhibited by apocynin) was increased in these patients. Figure 5D and 5E shows that NOX4 and NOX5 were present in human RA samples but were unaffected by permanent AF. In contrast, the protein level of mitochondrial complexes I through V was increased in the presence of permanent AF (Figure 6A), and inhibition of mitochondrial complex 1 with rotenone significantly decreased superoxide production in these patients (Figure 5A). Inhibition of NOS with L-NAME caused a significant reduction in superoxide production in RA tissue from patients in permanent AF (Figure 5A); this finding was associated with a reduction in BH4 concentration (Figure 6B)

### Table. Patients’ Clinical and Demographic Characteristics

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<th>Postoperative AF</th>
<th>Permanent AF</th>
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<td>72 (56)</td>
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<td>15 (58)</td>
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<td>14 (19)</td>
<td>4 (12)</td>
<td>11 (42)</td>
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<td>Surgical procedure, n (%)</td>
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<td>32 (100)</td>
<td>18 (69)</td>
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<td>0 (0)</td>
<td>8 (31)*</td>
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<td>59 (82)</td>
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<td>7 (22)</td>
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<td>3±1.6</td>
<td>4±2</td>
<td>5.5±2.2</td>
</tr>
</tbody>
</table>

Medications, n (%):
- Aspirin: 83, 56 (78), 21 (66), 6 (23)
- β-blockers: 75, 42 (58), 19 (60), 14 (54)
- Statins: 85, 51 (71), 22 (69), 12 (46)
- Calcium antagonists: 25, 13 (18), 6 (19), 6 (23)
- ACEIs and ARBs: 87, 46 (64), 20 (62), 21 (81)
- Diuretics: 50, 25 (35), 10 (31), 15 (58)

### Note

1. BH4: tetrahydrobiopterin
2. BH2: 4-hydroxybiopterin
3. BH2: 2-hydroxybiopterin
4. 2-OHE: 2-oxo-6-hydroxyethane
5. L-NAME: Nω-nitro-L-arginine methyl ester
6. NADPH: nicotinamide adenine dinucleotide phosphate
7. NOS: nitric oxide synthase
8. NOX: NADPH oxidase
9. AP: apocynin
10. DPI: dihydroethidium

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and a lower ratio of BH$_4$ to BH$_3^+$-bioppterin (Figure 6C) in the absence of changes in BH$_3$ or bioppterin (Figure 6D). Neither eNOS phosphorylation (Figure 6E) nor arginase activity (Figure 6F) was affected in patients with permanent AF.

In summary, among patients who developed AF after cardiac surgery, RA NADPH oxidase protein expression and activity were increased in the absence of differences in CD18 staining, suggesting that NADPH oxidase activity in atrial myocytes may play a role in the new onset of AF and in AF-induced early electric remodeling. In contrast, in patients with permanent AF, mitochondrial oxidases and uncoupled NOS activity accounted for the increase in RA superoxide release (as was observed in the goat in the presence of atrial structural remodeling secondary to 6-month AF or AVB). These data also indicate that an increase in NADPH oxidase activity is neither sufficient nor necessary to induce NOS uncoupling in the RA myocardium and in the absence of a significant reduction in tissue BH$_4$ concentration.

**Effect of Ex Vivo Atorvastatin in Right Atrial Samples From Patients With Postoperative or Permanent Atrial Fibrillation**

We compared the mevalonate-reversible and cholesterol-independent effects of statins in RA samples from patients in SR who developed AF after cardiac surgery and in patients with permanent AF. Rac1 activity was increased in RA samples from patients who developed postoperative AF (Figure 7A and 7B). Incubation with atorvastatin caused a mevalonate-reversible reduction in superoxide production in this group (Figure 7C) that was associated with a decrease in Rac1 activity (Figure 7B) and in the membrane translocation of the cytosolic p47$^{phox}$ and p67$^{phox}$ subunits of NADPH oxidase (Figure 7D). In contrast, in patients with permanent AF, atorvastatin did not affect RA superoxide production, Rac1 activity, or p47$^{phox}$ and p67$^{phox}$ translocation (Figure 7A through 7D) or restore normal NOS activity (as assessed by the absence of a mevalonate-reversible reduction in the L-NAME-inhibitable superoxide production; Figure 7E). In keeping with these findings, atorvastatin also failed to induce mevalonate-reversible changes in the RA BH$_4$ concentration or in the ratio of BH$_4$ to BH$_3^+$-bioppterin (Figure 8A through 8D). These differences in the effect of atorvastatin between groups were not associated with changes in HMG CoA-reductase protein level (Figure 7F). In summary, these data show that statins effectively reduce RA Rac1 activity and superoxide production in patients who develop postoperative AF but do not have such effects on the RA NO-redox balance in patients with permanent AF.

**Discussion**

The present study demonstrates that the mechanisms responsible for the myocardial NO-redox imbalance in AF differ between the RA and LA and with the duration of AF. These differences may well influence the response to therapeutic strategies that are targeted at signaling pathways upstream of ion channels. This conclusion is supported by a number of novel findings. First, the NADPH oxidase activity and the protein level of the cytochrome-forming subunits of the
oxidase (ie, NOX2 and p22phox) were significantly increased in the LA myocardium of goats after 2 weeks of AF and in RA samples from patients who developed AF after cardiac surgery in the absence of differences in CD18 staining. In contrast, in the RA of patients with persistent AF and in the RA and LA of goats after 6-month AF or AVB, the expression of mitochondrial oxidase and ROS production accounted for the increase in superoxide production. Under these conditions, NADPH oxidase activity and protein and gene expression of p22phox, NOX2, NOX4, and NOX5 did not differ from SR. Second, uncoupled NOS activity was detected only in the RA (in patients with persistent AF and goats with 6-month AF or AVB), where it was associated with a reduction in the availability of the NOS critical cofactor BH4 and/or with an increase in arginase activity. The reduction in the ratio of BH4 to BH2 \( \text{biopterin} \) that accompanied the increase in ROS production from NADPH oxidases was not sufficient to cause NOS uncoupling in the absence of a significant reduction in tissue BH4 concentration; the latter was observed only in the presence of longstanding AF. Third, atorvastatin caused a mevalonate-reversible inhibition of RA Rac1 activity, p67phox, and p47phox membrane translocation, and superoxide production in patients who developed AF after cardiac surgery, but it did not affect RA superoxide production, NOS uncoupling, or BH4 content in patients with permanent AF.

Together, these findings indicate that upregulation of NADPH oxidases in the atrial myocardium is an early but transient event in the natural history of AF and that a shift in the atrial sources of ROS with the duration and substrate of AF may influence the antiarrhythmic efficacy of statins.

### Reactive Oxygen Species and Atrial Fibrillation–Induced Electric Remodeling: Effects of Statins

Reactive oxygen species have been shown to affect myocardial electrophysiology and contractile properties. In the atrial myocardium, ROS-mediated injury leads to altered myofibrillar energetics \(^3\) and a reduction in the atrial effective refractory period, which, in turn, increases AF vulnerability. \(^8\) A causal relationship between myocardial oxidative stress and AF-induced electric remodeling was first suggested by Carnes et al. \(^8\) who observed that high doses of vitamin C prevented myocardial oxidative injury and the reduction in atrial effective refractory period induced by 48 hours of rapid atrial pacing in dogs. However, later studies did not confirm a beneficial effect of high-dose vitamin C (alone or in combination with vitamin E) in dogs after 1 week of rapid atrial pacing \(^9\) and reported that short-term administration of statins...
was highly effective in preventing atrial electric remodeling and AF in the same model.9 Statins have been shown to reduce superoxide formation by NADPH oxidases in human myocardial tissue by inhibiting Rac1 activity.16,17 Rac1-regulated production of superoxide by NADPH oxidase in nonphagocytic cells has proved to be both an important determinant of the low-intensity basal release of superoxide and a core element in the transduction of cardiovascular signals, including angiotensin II, endothelin-1, and cytokines.30 We have previously reported that NADPH-stimulated superoxide production in the RA myocardium (but not plasma markers of protein or lipid oxidation) is an independent predictor of the occurrence of postoperative AF in patients undergoing coronary artery bypass surgery.10 Together, these findings suggest that rapid antioxidant effects of statins, mediated by inhibition of NOX2-containing NADPH oxidases, may account for the antiarrhythmic effects of short-term statin treatment in patients undergoing cardiac surgery11 and in animal models of rapid atrial pacing.9 Consistent with this hypothesis, we found that an NADPH oxidase–dependent increase in atrial superoxide production was present in the goat LA myocardium after 2 weeks of pacing-induced AF and in the RA of patients in SR who develop AF after cardiac surgery. Whether superoxide production from NADPH oxidase affects the atrial effective refractory period (particularly in the LA) and, by doing so, promotes the initiation and maintenance of AF remains to be demonstrated. Leukocyte infiltration is not likely to be the cause of the increased NOX2 expression and activity under these conditions, as indicated by the lack of differences in atrial CD18 staining between AF groups in both goats and humans. These data are in partial agreement with our previous finding that NADPH oxidase, mitochondrial oxidases, and uncoupled NOS contributed to the increased RA superoxide production in 15 patients with paroxysmal or longstanding AF.6 In those patients, we found that the contribution of NADPH oxidases was largest in the subgroup with paroxysmal AF (unpublished observation). In the present study, mitochondrial oxidases and uncoupled NOS activity accounted for the increase in atrial superoxide release in patients with permanent/longstanding AF and in goats after 6-month AF or AVB. This suggests that, under these conditions, atrial ROS production may be secondary to atrial structural remodeling rather than to the presence of AF per se. Nevertheless, atrial structural remodeling (eg, secondary to AVB) provides a strong substrate for AF,21 and the increase in atrial ROS production might be a contributing factor. A recent study in a dog model of pacing-induced left ventricular failure has provided the first evidence that this may be the case; oral supplementation of BH4 and L-arginine in these animals prevented LA NOS uncoupling and electric remodeling and significantly decreased AF inducibility.32

Figure 6. The protein level of mitochondrial complexes I through V was increased in the right atrial tissue from patients with permanent atrial fibrillation (Pe-AF; A), whereas BH4 (B) and the ratio of BH4 to BH2+biopterin (B; C) were significantly reduced in the absence of changes in BH2 and biopterin (D). Total endothelial nitric oxide synthase (eNOS) and its Ser-1177 and Thr-495 phosphorylated fractions (E) and arginase activity (F) were unaffected by Pe-AF. Data are expressed as mean±SEM. VDAC indicates voltage-dependent anion channel; SR, sinus rhythm. *P<0.05, **P<0.01, ***P<0.001 vs SR, unpaired Student t test (n=12 to 69).
We cannot categorically exclude a contribution of NOX4- and NOX5-containing NADPH oxidases to the increase in RA superoxide production observed in the presence of persistent AF, particularly because NOX4 activity might also be stimulated by Rac1. However, as shown in Figure 7, Rac1 activity was not increased in RA samples from patients with persistent AF, and atorvastatin did not cause a mevalonate-reversible reduction in superoxide production in

Figure 7. Effect of ex vivo atorvastatin on Rac1 and NADPH oxidase activity, and nitric oxide synthase uncoupling in right atrial homogenates from patients who developed postoperative atrial fibrillation (postAF) and those with permanent AF (Pe-AF). Atrial Rac1 activity was increased in patients who developed postoperative AF (postAF) compared with patients with permanent AF (Pe-AF; A and B). Atorvastatin caused a mevalonate-reversible inhibition of Rac1 activity (A and B), NADPH-stimulated superoxide production (C), and p47phox and p67phox membrane translocation (D) in postAF. In contrast, atorvastatin did not induce a mevalonate-reversible inhibition of total (C) or N-nitro-L-arginine methyl ester (L-NAME)-inhibitable superoxide production (E) in patients with Pe-AF. HMG-CoA reductase protein level did not differ between groups (F). All data are expressed as mean±SEM. *P<0.05 vs postAF; †P<0.05 for the mevalonate-reversible effect of atorvastatin; †P<0.05 vs untreated group, 1-way ANOVA (A through E) and unpaired Student t test (F) (n=8 to 19).

Figure 8. Atorvastatin (ATV) did not affect atrial BH4 concentration in either group (A and B) but caused a significant reduction in BH2 in postoperative atrial fibrillation (postAF) patients (C), resulting in a nonsignificant trend toward an increase in the ratio of BH4 to BH2+biopterin (B) in this group (D). All data are expressed as mean±SEM. MVA indicates mevalonate. *P<0.05 vs postAF; †P<0.05 vs untreated group, 1-way ANOVA (n=8 to 9).
this group, in agreement with studies indicating little or no beneficial effect of statins in the secondary prevention of AF or in disease states (eg, heart failure) associated with atrial structural remodeling. It should be noted that our studies in human atrial tissue are limited to samples of the RA appendage. Although the similarities in the findings obtained in human and goat RA myocardium in the presence of longstanding AF suggest that the goat is a representative model of human AF, we cannot be certain that the human LA myocardium would show the same results.

Nitric Oxide Synthase Uncoupling Contributes to the Nitric Oxide–Redox Imbalance in Atrial Fibrillation

Uncoupling of RA NOS activity in the presence of longstanding AF or AVB provides another mechanism contributing to the NO-redox imbalance in the fibrillating atrial myocardium. This phenomenon has been described extensively in the vascular endothelium of animal models of hypertension, diabetes mellitus, and left ventricular hypertrophy. It occurs in response to a number of mechanisms including reduced availability of the NOS cofactor BH4, depletion of the NOS substrate l-arginine owing to increased consumption of this amino acid by competing enzymes such as arginases, and reduced eNOS phosphorylation on Thr. Our findings indicate that a reduction in BH4 levels and/or an increase in arginase activity may account for the uncoupling of NOS activity in the RA myocardium in the presence of longstanding AF or AVB. Because the Nm of BH4 and the of its oxidized product, BH2, for eNOS are similar, it has been suggested that BH2 would compete with BH4 for binding to eNOS, thus promoting eNOS uncoupling and superoxide production. Our findings do not support this hypothesis and indicate that a reduction in the ratio of BH4 to BH2 per se (as observed in patients who developed postoperative AF) does not lead to NOS uncoupling in the human atrial myocardium in the absence of a significant decrease in tissue BH4 content.

Conclusions

Our findings demonstrate for the first time that the mechanisms responsible for the NO-redox imbalance in the fibrillating atrial myocardium evolve with the duration of AF and the development of atrial structural remodeling and imply that inhibition of NADPH oxidase activity by drugs such as statins may be effective only in preventing early AF-induced atrial electric remodeling or new-onset AF (particularly in the presence of inflammation and high levels of circulating cytokines such as after cardiac surgery and cardiopulmonary bypass). Whether agents that restore atrial NO bioavailability (eg, BH4 and folate supplementation) or reduce mitochondrial ROS production will be able to promote the maintenance of SR in patients with atrial structural remodeling remains to be established.

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Disclosures

None.
Atrial fibrillation (AF) is a very common arrhythmia, and its therapy remains a challenge. There is considerable interest in developing treatment strategies that target mechanisms upstream of ion channel modifications; however, whereas ion channel modifications are the common denominator of virtually all types of AF, the myocardial signaling upstream of atrial electric and structural remodeling differs with the stage and substrate of AF, demanding a more refined ad hoc approach to the prevention and management of this arrhythmia. Here, we show that the mechanisms responsible for the nitric oxide–redox imbalance in the fibrillating atrial myocardium change with the duration of AF and the development of atrial structural remodeling. Upregulation of atrial NOX2 activity and expression is an early but transient event in the natural history of AF and may be causally linked to both new-onset AF and early AF-induced atrial remodeling. Once AF becomes established and atrial structural remodeling ensues, the oxidase systems underlying the increase in reactive oxygen species shift from NOX2 to mitochondrial oxidases and uncoupled nitric oxide synthases. Ex vivo atorvastatin induces a mevalonate-reversible inhibition of atrial Rac1 and NOX2 activity in patients who developed AF after cardiac surgery, but it does not affect atrial reactive oxygen species production and nitric oxide synthase activity in patients with permanent AF. These findings imply that NOX2 inhibition by drugs such as statins may be effective only in preventing new-onset AF or early AF-induced electric remodeling of the atrial myocardium.
Atrial Sources of Reactive Oxygen Species Vary With the Duration and Substrate of Atrial Fibrillation: Implications for the Antiarrhythmic Effect of Statins
Svetlana N. Reilly, Raja Jayaram, Keshav Nahar, Charalambos Antoniades, Sander Verheule, Keith M. Channon, Nicholas J. Alp, Ulrich Schotten and Barbara Casadei

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SUPPLEMENTAL MATERIAL

Methods

Measurements of superoxide production: Atrial myocardial samples were carefully rinsed of blood, snap frozen in liquid nitrogen and stored at -80°C. Basal or NADPH (100 µmol/L)-stimulated superoxide production was measured in homogenized tissue by using two independent methods:

(1) Lucigenin-enhanced chemiluminescence by using a single tube luminometer (Berthold FB12) modified to maintain the sample temperature at 37°C, as described previously. Low concentrations (5 µmol/L) of lucigenin were used in all our experiments, as validated previously in human atrial tissue.

(2) Detection of 2-hydroxyethidium (2-OHE). Detection of 2-OHE was carried out as described previously. Atrial homogenates were incubated with DHE (50 µMol/L) for 15 minutes at 37°C followed by the HPLC analysis of the level of 2-OH E.

All results are shown as the tiron (10-100 mmol/L)-inhibitable fraction of 2-OHE or lucigenin-enhanced chemiluminescence.

Biopterin measurements. Chromatographic analysis of BH4, 7,8-dihydrobiopterin (BH2) and biopterin content in homogenized atrial tissue using HPLC were carried out as described previously. Briefly, frozen atrial tissue was homogenized in ice-cold resuspension buffer (50 mMol/L phosphate buffered saline, 1 mmol/L dithioerythritol, 1 mMol/L EDTA, pH 7.4), using a pre-cooled Polytron® probe (PT2100 Kinematika®, Switzerland). Following centrifugation (twice for 15 min at 13,000 rpm and 4°C), supernatants were mixed with an ice-cold acid
precipitation buffer (1 mmol/L phosphoric acid, 2 Mol/L TCA, 1 mmol/L dithioerythritol).
Samples were vigorously mixed and then centrifuged for 5 minutes at 13,000 rpm and 4°C,
followed by HPLC analysis of BH4, BH2 and biopterin content. All processing steps were
performed on ice and in a batch design, with a maximum of 12 samples per batch.

Arginase activity. Arginase activity in homogenized atrial tissue was measured as the nor-
NOHA-inhibitable fraction of L-ornithine. Homogenates were incubated with $^{14}$C L-arginine
(1.85 MBq/ml, Amersham Biosciences UK Ltd., UK) in the presence or absence of nor-NOHA
at 37°C for 4 hours. After deproteinasation, samples were analyzed by HPLC. Standards of $^{14}$C-
labelled L-arginine and L-ornithine (Amersham Bioscience UK Ltd.) were used to determine the
eluting time. Chromatographic peaks were integrated, adjusted according to their size in control
samples and expressed as a proportion of total $^{14}$C counts for each sample.

Immunoblotting. Antibodies were used to detect the expression of p22phox (a kind gift from Prof
A. Segal and Dr F.B. Wientjes, UCL, UK), NOX2 (a kind gift from Prof A. Segal, UK, and Dr
M. Queen, USA), NOX4 (a kind gift from Prof A. Shah, UK), NOX5 (Santa Cruz, USA) p47phox,
p67phox (Upstate, USA), Rac1 (Millipore, USA), mitochondrial complexes I-V (MitoScience Inc.,
USA), HMG-CoA reductase (a kind gift from Dr Gene Ness, University of South Florida, USA),
CD18 (R&D Systems, USA), and eNOS (BD Transduction Lab., USA) and its serine 1177 and
threonine 495 phosphorylated fractions (Cell Signalling, USA).

Atrial homogenates were prepared in tissue lysis buffer containing a protease inhibitor cocktail
(Roche, UK). Immunodetection of the primary antibodies was performed with peroxidase-
conjugated secondary anti-rabbit or anti-mouse antibodies (Promega, USA), visualized with anti-
IgG horseradish peroxidase and enhanced chemifluorescence (Amersham Bioscience UK Ltd.),
and quantified relative to GAPDH or VDAC (for mitochondrial proteins, Abcam).
**Quantitative real time RT-PCR** was used to compare the expression of the primary transcripts of NOX2, NOX4, NOX5 using the following TaqMan probes (Applied Biosystems): human NOX2 (NM_000397.3, product # Hs00166163_m1), human NOX4 (NM_016931.3, product # Hs00418356_m), human NOX5 (NM_024505.2; product # Hs00225846_m1) & human GAPDH (NM_002046.3, product # 4333764T, served as a loading exogenous control). All primers and probes spanned two introns. RT-PCR was performed using an iCycler IQ real-time detection system (BioRad Laboratories, USA) in 96-well optical plates (Applied Biosystems, USA). For each 30 μl reaction, 13.5 μl of cDNA (100 ng/μl) was mixed with 15 μl TaqMan universal master mix and 1.5 μl TaqMan gene expression assays. The reaction conditions were as follows: 50°C for 10 minutes, then 95°C for 10 minutes followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Results were expressed as ΔCt, corresponding to the difference between the Ct of the gene of interest and the Ct of the gene used to normalize the results (here the GAPDH gene).

**Measurement of Rac1 activity.** Rac1 activation was evaluated by an affinity precipitation assay using the PAK1-PBD conjugated glutathione agarose beads, according to the manufacturer’s instructions (Millipore, Temecula, USA). Human right atrial samples were homogenized on ice in 500 μL of lysis buffer. Pull-down of GTP-bound Rac1 was performed by incubating tissue lysates (600 μg) with GST fusion protein corresponding to the p21-binding domain of PAK-1 bound to glutathione-agarose for 1 h at 4 °C. The beads were washed three times with lysis buffer, and the protein bound to the beads was eluted with Laemmli buffer and analyzed for the amount of GTP-bound Rac1 by immunoblotting using a Rac1 monoclonal antibody. Rac1 activation was defined by the GTP-Rac1:total Rac1 ratio.
Membrane translocation of the p67phox and p47phox subunit of NADPH oxidase was evaluated in the presence or absence of ex vivo application of atorvastatin (20 µmol/L for 60 minutes at 37°C). Briefly, right atrial tissue was homogenized in ice-cold HEPES buffer (in mmol/L: 20 HEPES, 150 NaCl, and 1 EDTA, pH 7.4) containing protease inhibitor cocktail (Roche, UK). Cell nuclei and unbroken cells were removed by centrifugation of the homogenates at 2,800 g at 4°C for 20 min. Further centrifugation of the supernatants at 100,000 g for 60 min at 4°C allowed the separation of the membrane proteins in the pellets, which were re-suspended in HEPES buffer containing 1% Triton for 20 min on ice and used to assay the p47phox and p67phox proteins by immunoblotting.

Immunostaining for CD18 was performed in human RAA and goat LA tissue blocks embedded in OCT media, which were cut into 10 µm sections using cryotome (-28°C). Following fixation in pre-cooled acetone (-20°C) for 10 min, the sections were air-dried and rinsed in phosphate buffered saline (PBS). After a blocking step with 10% normal horse serum for 30 min at room temperature, the primary anti-CD18 (R&D Systems, USA) and anti-α-actinin antibodies were applied and incubated overnight at 4°C. Following a rinsing step with PBS, secondary Alexa Fluor antibodies (Invitrogen, USA) were applied to the sections for 1 hour at room temperature, and rinsed afterwards with PBS. The slides were finished with coverslips using mounting solution and dried overnight at room temperature in a dark. Imaging was performed on the following morning using a Zeiss confocal microscope.
References


Supplementary Figure 1. The eNOS phosphorylated fractions did not differ between the right (RA) and left atrium (LA) or between groups, although total eNOS was reduced in the left atrium after 6M-AF or AVB (A).

The protein level of antioxidant enzymes did not differ between atria or between groups. There was a trend for MnSOD to be lower in the right atrium in the 6M-AF group (B). Abbreviations are as defined in Figure 1. Data are expressed as mean of n=6-12 SEM. * P < 0.05 vs. SR.
Supplementary Figure 1. The eNOS phosphorylated fractions did not differ between the right (RA) and left atrium (LA) or between groups, although total eNOS was reduced in the left atrium after 6M-AF or AVB (A).

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