Tetrahydrobiopterin depletion and NOS2 uncoupling contribute to heart failure-induced alterations in atrial electrophysiology

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Aims
Heart failure is a common antecedent to atrial fibrillation; both heart failure and atrial fibrillation are associated with increased myocardial oxidative stress. Chronic canine heart failure reduces atrial action potential duration and atrial refractoriness. We hypothesized that inducible nitric oxide synthase 2 (NOS2) contributes to atrial oxidative stress and electrophysiologic alterations.

Methods and results
A 16-week canine tachypacing model of heart failure was used (n = 21). At 10 weeks, dogs were randomized to either placebo (n = 12) or active treatment (n = 9) with NOS cofactor, tetrahydrobiopterin (BH4, 50 mg), and NOS substrate (L-arginine, 3 g) twice daily for 6 weeks. A group of matched controls (n = 7) was used for comparison. Heart failure increased atrial NOS2 and reduced atrial BH4, while L-arginine was unchanged. Treatment reduced inducible atrial fibrillation and normalized the heart failure-induced shortening of the left atrial myocyte action potential duration. Treatment increased atrial BH4 while L-arginine was unchanged. Treatment did not improve left ventricular function or dimensions. Heart failure-induced reductions in atrial BH4 resulted in NOS uncoupling, as measured by NO and superoxide anion production, while BH4 and L-arginine treatment normalized NO and O2·-. Heart failure resulted in left atrial oxidative stress, which was attenuated by BH4 and L-arginine treatment.

Conclusion
Chronic non-ischaemic heart failure results in atrial oxidative stress and electrophysiologic abnormalities by depletion of BH4 and uncoupling of NOS2. Modulation of NOS2 activity by repletion of BH4 may be a safe and effective approach to reduce the frequency of atrial arrhythmias during heart failure.

Keywords
Heart failure • Arrhythmia mechanisms • Oxygen radicals • Nitric oxide

1. Introduction
Heart failure and atrial fibrillation are common co-existing disease states, and heart failure results in a 4.5- to 5.9-fold increase in the risk of atrial fibrillation. Patients with advanced heart failure who develop atrial fibrillation are at a significantly increased risk of death. Thus, the identification of factors that predispose to the development of atrial fibrillation during heart failure, and the evaluation of specifically targeted pharmacologic interventions have the potential to reduce morbidity and mortality significantly in this patient population. There are multiple biochemical alterations occurring in the myocardium during heart failure. During heart failure, multiple sources of oxidative stress, including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (NOS), have been implicated in the disease process. Induction of NOS2 (inducible NOS or iNOS) has been described in models...

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of heart failure, as well as in human heart failure.\textsuperscript{4,5} NO synthases become functionally uncoupled under conditions of cofactor (i.e. tetrahydrobiopterin, BH\textsubscript{4}) depletion, which shifts the enzymatic activity from NO production towards superoxide anion (O\textsubscript{2}·−) production.\textsuperscript{6,7} Thus, induction of NOS2 in the myocardium with concurrent depletion of BH\textsubscript{4} can serve as a source of free radicals and oxidative stress.

Oxidative stress is known to contribute to the pathogenesis of atrial fibrillation.\textsuperscript{8–11} We have previously reported that in chronic (4-month) canine heart failure, there is a substrate for sustained atrial fibrillation, and that heart failure-induced abnormalities in atrial myocyte electrophysiology are partially improved by treatment with N-acetylcysteine, a precursor to the reducing equivalent, glutathione.\textsuperscript{12} In the present study, we tested the hypothesis that induction and uncoupling of atrial NOS2 in this chronic model of canine heart failure results in atrial oxidative stress and electrophysiologic abnormalities.

2. Methods

Additional detailed methods are in Supplementary material online.

2.1 Animal model and in vivo measurements

A total of 21 hound type dogs of either sex (2–3 years of age) had a right ventricular (RV) pacemaker lead implanted in the RV apex; pacing was performed to induce stable chronic heart failure as previously described\textsuperscript{12,13} Animals were given a pre-anesthesia dose of morphine (0.5–1 mg/kg intravenously) followed by isoflurane (5% by mask). After induction of anesthesia, dogs were rapidly intubated and received isoflurane 2–3% by inhalation. Adequacy of anesthesia was assessed by monitoring heart rate. A bipolar pacing electrode was advanced in transeptally into the right ventricle using fluoroscopic guidance and affixed to a Prevail Medtronic pacemaker lead implanted subcutaneously in the neck. The incision was closed, and the dog permitted to recover for 7–10 days, receiving cefalexin (500 mg po bid for 2–3 days, up to 7–10 days if indicated).

Serial electrocardiograms (ECGs), 2D and M-mode echocardiograms were performed at baseline and during brief periods of sinus rhythm during the pacing protocols, during butorphanol sedation (0.5 mg kg\textsuperscript{-1}.im).\textsuperscript{9,12,13} ECG lead II was recorded (MP100WSW, Biopac Systems, Inc., Santa Barbara, CA, USA), and P-wave duration measurements were obtained from the average of 10 consecutive beats. The RV was paced at 180 b.p.m. for 2 weeks; 200 b.p.m. for the next 6 weeks, followed by 180 b.p.m. for the duration of the protocol (modified Prevail 8086 pacemakers, Medtronic, Inc., Minneapolis, MN, USA). Serial electrocardiograms and ECGs were obtained to assess cardiac structure and function.

For the last 6 weeks of the 4-month period, animals were randomly assigned to either active treatment (n = 9) with BH\textsubscript{4} (Schircks Laboratories, Switzerland) 50 mg po + l-arginine 3 g (GNC, Inc) given orally twice daily, or to placebo (n = 12). Doses were selected based on allometric dosing principles\textsuperscript{14} based on previously used doses of BH\textsubscript{4} and l-arginine.\textsuperscript{15,16} A group of age-matched control dogs (n = 7) were used as a comparator for all experiments.

At the end of the 4-month period, effective refractory periods (ERPs) were determined using a train of eight pacing stimuli at a cycle length of 300 ms, followed by an increasingly premature extrastimulus (5 ms decrement) delivered by a programmable stimulator (Medtronic Model 3325, Medtronic Inc.). The longest extrastimulus not propagated was defined as the ERP. All ERPs were determined at twice the diastolic pacing threshold. Inducibility of atrial fibrillation was assessed with three attempts (burst pacing at 10 Hz for 10 s) at 5 min intervals. After ERPs were measured, up to three attempts to induce atrial fibrillation were performed as previously described.\textsuperscript{9,17} Atrial fibrillation was defined as the absence of P-waves and irregular intervals between QRS complexes on the ECG. At the conclusion of each in vivo study, animals were euthanized, and the heart was rapidly excised for isolation of atrial tissues and myocytes.\textsuperscript{12} All animal procedures were approved by the Ohio State University Institutional Animal Care and Use Committee (Protocol 2010A00000103). The investigation conforms with the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Tissue and myocyte isolation

Immediately after flushing the heart with cardioplegia solution,\textsuperscript{18} left atrial tissue was collected from the region just adjacent to the appendix for subsequent tissue analyses. After collection, left atrial tissue was snap-frozen in liquid nitrogen and stored either at −80°C or in liquid nitrogen, or embedded in optimal cutting temperature medium and stored at −80°C for subsequent analyses. Myocytes were isolated from the left atrial appendage as previously described\textsuperscript{13} by cannulation and perfusion of the left circumflex artery. After the heart was collected in cold saline, it was rapidly flushed via the coronary ostia with cold cardioplegia solution. The circumflex artery was cannulated for perfusion on a Langendorff apparatus with Krebs–Henseleit buffer containing collagenase (Worthington Type II). After perfusion was completed, the left atrial appendage was minced and placed into 5 mL of perfusate in a shaking water bath at 37°C for 5–15 min. Atrial myocytes were filtered and then centrifuged twice with incubation buffer (IB) to remove collagenase, prior to suspension in storage buffer. The myocytes were stored at room temperature in a standard IB solution containing (in mM) NaCl 118, KCl 4.8, MgCl\textsubscript{2} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, gluta mine 0.68, glucose 10, pyruvate 5, CaCl\textsubscript{2} 1, along with 1 μmol L\textsuperscript{-1} insulin, and 1% BSA until used. This typically yielded 40–60% rod-shaped myocytes with staircase ends and sharp margins. All electrophysiologic data were collected within 8 h of myocyte isolation.

2.3 Cellular electrophysiology

Myocytes were placed in a laminin-coated cell chamber (Cell Microcontrols, Norfolk, VA, USA) and superfused with solution containing (in mM): 135 NaCl, 5 MgCl\textsubscript{2}, 5 KCl, 10 glucose, 1.8 CaCl\textsubscript{2}, 5 HEPES, pH adjusted to 7.40 with NaOH, at a temperature of 35 ± 0.5°C. Borosilicate glass micropipettes (1.5–3 MΩ) were filled with pipette solution containing (in mM): 100 K+ aspartate, 40 KCl, 5 MgCl\textsubscript{2}, 5 EGTA, 5 HEPES, pH adjusted to 7.2 with KOH. Action potential (AP) durations (APDs) were measured from the average of the last 10 (steady-state) APs, obtained during a train of 25 APs at each stimulation rate.

2.4 Interstitial fibrosis

Tissue was collected just proximal to the left atrial appendage for assessment of interstitial fibrosis. Masson’s trichrome was used to define the % area of fibrosis.\textsuperscript{17} The tissue sections were visualized using a Nikon microscope (TE2000-U, Japan) and areas occupied by blue pixels (fibrosis) were quantified using MetaMorph image analysis software (Molecular Devices, CA, USA).

2.5 NOS, NOS activity, and modulators of NOS activity

Expression of NOS2 was assessed by immunoblot analysis. Left atrial tissue homogenate proteins (40 μg) were subjected to SDS 4–20% PAGE, blotted onto nitrocellulose membranes (Bio-Rad Hercules, CA, USA), and probed with anti-NOS2 antibodies (1:1000) from Millipore (Bedford, MA, USA). GAPDH levels were measured as an internal control with anti-GAPDH (1: 2500) antibody (Abcam, Cambridge, MA, USA). Blots were developed with Super Signal West Pico (Pierce), scanned, and quantified by using an Image J (NIH) and Origin 7.0 (OriginLab, Northampton, MA, USA) software.
Total RNA was isolated from control, heart failure, and heart failure + BH4, and l-arginine-treated canine left atrial heart specimens using TriZOL (Invitrogen) to assess NOS2 RNA expression.

Left atrial tissue was imbedded in 2′,3′-diolethidium (DHE) fluorescence, as previously described. After cryosectioning, 10 μM DAF-FM diacetate was applied to the tissues for 8 C in a light-protected chamber, washed with PBS to remove any unbound DHE, fixed with mounting media, and imaged with a fluorescence microscope (Nikon TE 300 Model, Tokyo, Japan) with a fluorescence microscope (Nikon TE 300) and imaged with a fluorescence microscope (Nikon TE 300 Model, Tokyo, Japan) with a fluorescence microscope (Nikon TE 300) with a rhodamine filter (green excitation, 550 nm; red emission, 570 nm) and imaged with a fluorescence microscope (Nikon TE 300). The fluorescence intensity, which positively correlated with the amount of NO generation, was quantitatively determined using the MetaMorph image analysis software (Molecular Devices), as previously described.21

Left atrial arginines, BH4, and dihydrobiopterin (BH2) were assayed in tissue homogenates, prepared from snap-frozen samples, using previously described HPLC techniques.22

2.6 Electron paramagnetic resonance spectroscopy

The atrial tissue samples were flash frozen and stored in liquid nitrogen for electron paramagnetic resonance (EPR) analysis to measure radical and paramagnetic species. Both semi-quinone radical and Fe-S centres were quantified using previously described methods. Each EPR sample was prepared by transferring the frozen heart tissue (80–370 mg) into a ceramic mortar pre-chilled with liquid nitrogen. The tissue was then crushed in liquid nitrogen using a pestle.24 The tissue in liquid N2 was then loaded into a finger Dewar containing liquid nitrogen.22

Left atrial arginines, BH4, and dihydrobiopterin (BH2) were assayed in tissue homogenates, prepared from snap-frozen samples, using previously described HPLC techniques.22

Table 1  Echocardiographic parameters in heart failure animals obtained at baseline, pre-randomization to either placebo or BH4 + L-arginine, and after 6 weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>HF-pre-treatment</th>
<th>HF-placebo post-treatment</th>
<th>Baseline</th>
<th>HF-pre-treatment</th>
<th>HF-BH4 + arginine post-treatment</th>
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<tbody>
<tr>
<td>Left ventricle</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LV internal diameter-diastole (cm)</td>
<td>2.8 ± 0.2</td>
<td>3.6 ± 0.8*</td>
<td>3.8 ± 0.3*</td>
<td>3.0 ± 0.1</td>
<td>3.7 ± 0.1*</td>
<td>4.1 ± 0.1*†</td>
</tr>
<tr>
<td>LV internal diameter-systole (cm)</td>
<td>1.7 ± 0.1</td>
<td>2.9 ± 0.3*</td>
<td>3.2 ± 0.3*</td>
<td>1.7 ± 0.1</td>
<td>3.0 ± 0.1*</td>
<td>3.5 ± 0.4*†</td>
</tr>
<tr>
<td>Fractional shortening (%)</td>
<td>43.5 ± 1.5</td>
<td>21.2 ± 2.7*</td>
<td>18.1 ± 1.5*</td>
<td>44.8 ± 1.6</td>
<td>19.1 ± 1.0*</td>
<td>15.8 ± 0.7*</td>
</tr>
<tr>
<td>Left atrium</td>
<td></td>
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<tr>
<td>LA area: end-diastole (cm²)</td>
<td>4.4 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>5.8 ± 1.0</td>
<td>5.3 ± 0.4</td>
<td>6.0 ± 0.5</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>LA area: end-systole (cm²)</td>
<td>6.4 ± 0.4</td>
<td>7.0 ± 0.3</td>
<td>7.6 ± 1.1</td>
<td>7.4 ± 0.5</td>
<td>8.3 ± 0.5</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>Fractional area change (%)</td>
<td>31.2 ± 2.0</td>
<td>31.9 ± 1.9</td>
<td>25.6 ± 1.7*</td>
<td>30.6 ± 1.7</td>
<td>31.8 ± 2.5</td>
<td>25.9 ± 1.4*</td>
</tr>
</tbody>
</table>

HF=heart failure, LV-left ventricular, LA-left atrial.
*P < 0.05 vs. baseline.
†P < 0.05 vs. pre-treatment value.

2.7 Cellular electrophysiology

Perforated patch, whole cell patch clamp techniques were used, as previously described.12 Data acquisition was performed with Clampex (version 8.0 or 9.0) software (Axon Instruments Inc., Union City, CA, USA) and Axopatch 200A patch clamp amplifiers (Axon Instruments Inc.). Amphoterein B perforated whole cell patch clamp (36 ± 0.5°C) was used to minimize alterations in intracellular milieu during action potential recordings.

3. Results

As previously reported in this model, RV tachypacing resulted in left ventricular (LV) chamber dilation and impaired contractility, which was evident as increased LV chamber diameters, reduced LV posterior wall thickness, and reduced LV fractional shortening (P < 0.05; Table 1). Treatment with BH4 and l-arginine did not improve LV dimensions or function (P = NS vs. placebo; Table 1). There was a time-dependent reduction in left atrial contractility, assessed as fractional area change in the placebo-treated heart failure group (P < 0.04), which was similarly reduced in the BH4 and arginine-treated group (P < 0.05). There was reduced inducibility of atrial fibrillation (P < 0.05) in the BH4 and l-arginine co-supplemented group (0 of 9) compared with the placebo-treated heart failure group (6 of 12). The atrial ERP at a basic cycle length of 200 ms was longer in the BH4 and l-arginine co-supplemented relative to the placebo-treated heart failure group (Figure 1, P < 0.05), while the atrial ERPs at cycle lengths of 400 and 300 ms after BH4 and arginine supplementation (P = 0.14 and 0.09, respectively) were not significantly different. Atrial conduction velocity, assessed globally as P-wave duration, did not differ between the two heart failure groups.
groups at baseline. After 4 months of heart failure, the P-wave was significantly prolonged in both groups ($P < 0.05$), and did not differ between the groups ($P = \text{NS}$) (Figure 1).

Left atrial myocyte action potentials were significantly shorter in the placebo-treated heart failure group relative to control, at 50 and 90% repolarization (APD50 and APD90, respectively) at both 1 and 2 Hz (Figure 2). In contrast, in the BH4 and arginine-treated heart failure group, APD50 and APD90 did not differ from control values, and were significantly longer relative to the placebo-treated heart failure group (Figure 2).

Myocyte capacitance was assessed as a measure of cell size, and was increased in the placebo-treated heart failure (HF) group relative to controls (see Supplementary material online, Figure S1). Interstitial fibrosis was assessed and was markedly increased in the HF placebo group. The increase in left atrial fibrosis was attenuated by BH4 and arginine treatment (see Supplementary material online, Figure S2).

Heart failure induced a significant ~five-fold increase in the expression of NOS2 in left atrial tissue in comparison with controls; this was evident in both the placebo and BH4 and arginine-treated heart failure groups (Figure 3A, $P < 0.05$). The mRNA levels for NOS1, NOS2, and NOS3 did not differ among the three groups (see Supplementary material online, Figure S3). The heart failure-induced increase in the expression of NOS2 was accompanied by a reduction in left atrial [BH4] (Figure 3B), while treatment with BH4 and arginine supplements significantly increased left atrial [BH4]. The concentration of BH4 was less than the lower limit of detection in all groups. Left atrial [arginine] was unchanged by heart failure (Figure 3C) and remained unchanged by supplementation. The concentrations of left atrial asymmetric methylarginines (ADMA) did not differ among the three groups (data not shown).

Consistent with uncoupling of NOS2, immunofluorescence indicated that left atrial NO production was reduced in the placebo-treated heart failure group, while left atrial production of $\mathrm{O}_2^-$ was increased (Figure 4, $P < 0.05$). In contrast, treatment of heart failure animals with BH4 and arginine supplements resulted in a significant normalization of NO and $\mathrm{O}_2^-$ production towards control values (Figure 4).

EPR spectroscopy revealed an increase in both semi-quinone radicals and Fe-S centres in the left atrium of placebo-treated heart failure animals ($P < 0.05$, Figure 5A–C). In contrast, treatment of heart failure animals with BH4 and arginine supplements normalized both semi-quinone radicals and Fe-S centres to values that did not differ from controls.
4. Discussion

Our main findings are that chronic heart failure results in induction of NOS2 in the left atrium, which is associated with NOS cofactor depletion, NOS2 uncoupling, and consequent oxidative stress. These biochemical changes were associated with atrial electrophysiologic abnormalities, specifically inducibility of atrial fibrillation, attenuation of the atrial action potential duration, and the atrial ERP, physiologic alterations previously reported in this model of heart failure. Most significantly, chronic treatment with BH4 and arginine co-supplementation reduced susceptibility to atrial fibrillation, and attenuated left atrial electrophysiologic, structural, and biochemical abnormalities. This improvement apparently resulted from BH4 treatment rather than arginine treatment, as atrial arginine concentration was unchanged in any group. Collectively, these data suggest that manipulation of NOS activity by replacement of the cofactor, BH4,
may attenuate the atrial oxidative stress and electrophysiologic abnormalities occurring during chronic heart failure. Notably, the improvement in atrial electrophysiology did not result from improved LV function, as LV diameters and fractional shortening were not improved by manipulation of NOS activity.

Atrial fibrillation is promoted by shortening of atrial refractoriness and atrial action potential duration (recently reviewed by Nattel et al.26). In this study, we observed a shortening of left atrial action potential duration and refractoriness by heart failure of 4-month duration. However, heart failure has also been associated with atrial action potential duration prolongation or prolongation of atrial refractoriness.27–29 A recent study in patients with heart failure found a shortening of right atrial refractoriness.30 The discrepancies in the direction of change between our findings and previous reports could result from differences in duration and/or aetiology of heart failure, the site of study (right vs. left atrium), or the severity of heart failure.31 Further studies are needed to delineate the mechanism(s) underlying these divergent observations.

We recently reported that chronic canine heart failure results in attenuation of the atrial action potential duration and atrial ERP.12 Shortening of action potential duration and refractoriness are
Atrial NOS2 and heart failure

Cofactor was reduced in heart failure, and oral BH4 supplementation increased in NOS2 expression was unaltered by BH4 and arginine not increase concentrations further. While the heart failure-induced unchanged during chronic heart failure, and supplementation did not increase in superoxide anion based on the semi-quinone radical intensity (Figure 5B).

Recently, NOS2 has been shown to be increased in the left atria in a canine 2-week model of tachypacing-induced heart failure.38 NOS2 gene and protein expression has long been known to be minimal or absent in normal hearts and up-regulated in the ventricle during heart failure.39–41 In end-stage, human HF of mixed aetiology, NOS2 has been reported to be increased in all four heart chambers.39 The cellular source of NOS2 is not identified in many of these studies where tissue samples were used (as in the present study), although diffuse distribution in the cytosol of myocytes has been described in human heart failure.39 In the current study, NOS2 expression was increased in heart failure ~five-fold by western blot analyses; however, NOS2 mRNA was not increased in parallel. This inconsistency may be a result of a microRNA-mediated mechanism since these non-coding RNAs can inhibit gene expression by targeting mRNAs for translational repression and not mRNA degradation.42

NOS1 and NOS3 isoforms were not measurable by western blot analyses due to technical limitations; however, mRNA for NOS1 and NOS3 were unchanged between groups.

NOS activity may be modulated by ADMA concentrations. Elevated plasma ADMA concentrations have recently been associated with atrial fibrillation.43–45 However, circulating ADMA concentrations may not reflect tissue concentrations, and we did not find a difference in left atrial ADMA concentration between groups. Therefore, NOS2 activity in the present study was unlikely to be altered by ADMA.

We only studied chronic heart failure, and time dependence of altered NOS isoforms in heart failure has been reported.46 The contribution of NOS2 to other forms of atrial electrical abnormalities (e.g. during mitral regurgitation or atrial tachycardia or atrial fibrillation) is unknown. Alterations in NO have been reported to modulate (e.g. during mitral regurgitation or atrial tachycardia or atrial fibrillation) assessment by the Fe-S signal intensity (Figure 5C), which is attributable—at least in part—to the increase in superoxide anion based on the semi-quinone radical intensity (Figure 5B).

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to the fibrosis previously reported in a canine model of HF induced by 2 weeks of ventricular tachypacing. Treatment with BH4 and l-arginine attenuated the increase in fibrosis, suggesting either a reduction in the rate of acquisition of fibrosis or a reversal of fibrosis. The role of NOS modulation of fibrosis was previously examined in mice undergoing 4 weeks of transverse aortic constriction, and BH4 treatment was reported to reduce ventricular myocyte hypertrophy and fibrosis, an effect that was attributed to re-coupling NOS in myocytes and possibly fibroblasts.

P-wave duration has recently been suggested to have utility as a marker of atrial fibrillation risk. Increased atrial fibrosis is associated with slowing of atrial conduction velocity. While we found attenuation of interstitial fibrosis following BH4 and l-arginine treatment, it did not translate into normalization of the P-wave duration. It is possible that attenuation of fibrosis, without complete normalization, is insufficient to normalize the P-wave duration. Alternatively, other factors (e.g. connexin expression and/or distribution, sodium current) may have also contributed to HF-induced changes in P-wave duration. The present study was not designed to explicitly address structural remodelling, and further investigation is warranted to elucidate the mechanism(s) contributing to the reduced fibrosis and improved atrial conduction with BH4 and l-arginine treatment.

5. Summary and conclusions
Chronic heart failure results in biochemical alterations in the left atrium, which result in increased oxidative stress. Modulation of the activity of NO synthase by supplementation with the cofactor BH4 ameliorates atrial oxidative stress and normalizes atrial electrophysiology, reducing the inducibility of atrial fibrillation. Strategies to minimize NOS2 induction or modulate NOS2 activity may be effective in reducing atrial arrhythmias.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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