Oxidative Stress on Pulmonary Vein and Left Atrium Arrhythmogenesis

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Background: Oxidative stress and pulmonary veins (PVs) play critical roles in the pathophysiology of atrial fibrillation. The purpose of the present study was to investigate whether oxidative stress and antioxidant agents can change the electrophysiological characteristics of the left atrium (LA) and PVs.

Methods and Results: Conventional microelectrodes were used to record the action potentials (APs) in isolated rabbit PV and LA specimens before and after H₂O₂ administration with or without ascorbic acid or *N*-mercaptopyrionyl-glycine (N-MPG, a free radical ·OH scavenger). H₂O₂ (0.02 and 0.2 mmol/L) decreased the PV spontaneous rates from 2.0±0.1 Hz to 1.6±0.1 Hz, and 1.7±0.1 Hz (n=10, P<0.05), but H₂O₂ (2 mmol/L) increased PV spontaneous rates from 2.0±0.1 Hz to 2.8±0.2 Hz. H₂O₂ easily induced PV burst firing and early afterdepolarizations, but not in the LA. H₂O₂ shortened the AP duration and increased the contractile force to a greater extent in the LA than in PVs. In addition, the H₂O₂-induced PV burst firing and increasing spontaneous rates were suppressed or attenuated by pretreatment with ascorbic acid (1 mmol/L) or N-MPG (10 mmol/L).

Conclusions: H₂O₂ significantly changed the electrophysiological characteristics of PV and LA through activation of free radicals and may facilitate the occurrence of atrial fibrillation. (*Circ J* 2010; **74**: 1547–1556)

Key Words: Antioxidant; Atrial fibrillation; Hydrogen peroxide; Pulmonary vein

Atrial fibrillation (AF) is the most important sustained clinical arrhythmia and can induce cardiac dysfunction, and stroke and increase mortality.¹ Oxidative stress plays an important role in the pathophysiology of cardiovascular diseases.^{2–6} In addition, oxidative stress contributes to the genesis of AF.^{7–10} Oxidative modification of proteins was found in chronic AF patients.⁷ Moreover, it has been demonstrated that myocardial NAD(P)H oxidase significantly contributes to superoxide production in the human atrium during AF.⁹ Oxidative stress directly changes cardiac electrophysiological characteristics and also regulates calcium handling in cardiomyocytes,^{11,12} which may facilitate the genesis of AF. Through prevention of oxidative stress, antioxidant agents have been shown to have the potential to treat AF.^{13,14}

Pulmonary veins (PVs) have been shown to be the main sources of ectopic beats with the initiation of paroxysmal AF,^{15,16} and play a role in maintaining AF.¹⁷ PVs contain a mixture of working myocardium and pacemaker cells, which

can induce atrial arrhythmias.^{18–22} Because the risk factors (inflammatory cytokines, stretching, and angiotensin II) for AF have all been shown to increase oxidative stress and arrhythmogenesis,^{23–26} oxidative stress may increase PV and atrial arrhythmogenesis. Therefore, the purpose of the present study was to evaluate the electrophysiological effects of oxidative stress on PVs and the atrium, and investigate whether antioxidant agents can alter the oxidative stress on PVs and atrial electrical activity.

Methods

Rabbit PV and Left Atrium (LA) Tissue Preparations

The investigation conformed to the institutional Guide for the Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. As previously described, New Zealand rabbits (weighing 1.5–2.0 kg) were anesthetized with an iv injection of sodium pentobarbital (40 mg/kg).^{24,25} A mid-

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Figure 1. Effects of H₂O₂ on pulmonary vein (PV) spontaneous activity. **(A)** Average data before and after different concentrations (0.02, 0.2, and 2 mmol/L) of H₂O₂ on PV spontaneous activity (n=10). **(B)** H₂O₂ (0.02, 0.2, and 2 mmol/L)-induced PV burst firings and early afterdepolarizations (↓). *P<0.05, **P<0.01, ***P<0.005 vs baseline.

line thoracotomy was then performed and the heart with the lungs was removed. For dissection of the PVs, the LA was opened by an incision along the mitral valve annulus extending from the coronary sinus to the septum in Tyrode's solution with a composition of 137 mmol/L NaCl, 4 mmol/L KCl, 15 mmol/L NaHCO₃, 0.5 mmol/L NaH₂PO₄, 0.5 mmol/L

MgCl₂, 2.7 mmol/L CaCl₂, and 11 mmol/L dextrose. The PVs were separated from the atrium at the level of the LA–PV junction and separated from the lungs at the ending of the PV myocardial sleeves. One end of the preparations, consisting of the PVs and LA–PV junction, was pinned with needles to the bottom of a tissue bath. The other end (distal PV) was

Figure 2. Effects of H_2O_2 on action potential (AP) of the pulmonary veins (PVs) without spontaneous activity. **(A)** Superimposed tracings and **(B)** average data of the AP and contractility in PVs ($n=8$) before and after administration of different concentrations (0.02, 0.2, and 2 mmol/L) of H_2O_2 . **(C)** Examples of H_2O_2 (0.02, 0.2, and 2 mmol/L)-induced PV burst firings and early after-depolarizations (↓). * $P<0.05$, ** $P<0.01$, *** $P<0.005$ vs the baseline. APA, AP amplitude; APD_{20,50,90}, AP duration at a repolarization of 20%, 50%, 90% of the APA; RMP, resting membrane potential.

connected to a Grass FT03C force transducer with a silk thread. The adventitia or epicardial side of the preparations faced upwards. For LA experiments, the LA appendage (approximately $10 \times 5 \times 0.5$ mm) was isolated and prepared as described previously.²⁵ The PV and LA tissue strips were superfused at a constant rate (3 ml/min) with Tyrode's solution saturated with a 97% O_2 –3% CO_2 gas mixture. The temperature was maintained at 37°C , and the preparations were allowed to equilibrate for 1 h before electrophysiology assessment.

Electrophysiology and Pharmacology

The transmembrane action potential (AP) of the PVs and LA was recorded using machine-pulled glass capillary micro-electrodes filled with 3 mol/L of KCl. Preparations were connected to a WPI model FD223 electrometer under a tension of 150 mg. The electrical and mechanical events were shown simultaneously on a Gould 4072 oscilloscope and Gould TA11 recorder. The signals were recorded digitally with a 16-bit accuracy at a rate of 125 kHz. Electrical stimulus with

Figure 3. Effects of H₂O₂ on action potential (AP) morphology in the left atrium (LA). **(A)** Superimposed tracings and **(B)** average data of AP and contractility in LAs (n=10) before and after administration of different concentrations (0.02, 0.2, and 2 mmol/L) of H₂O₂. **(C)** H₂O₂ (2 mmol/L)-induced LA-triggered beats (↓). *P<0.05, **P<0.01, ***P<0.005 vs baseline. APA, AP amplitude; APD_{20,50,90}, AP duration at a repolarization of 20%, 50%, 90% of the APA; RMP, resting membrane potential.

a 10-ms duration and supra-threshold strength was provided by a Grass S88 stimulator through a Grass SIU5B stimulus isolation unit. Different concentrations of H₂O₂ (0.02, 0.2, and 2 mmol/L) were sequentially superfused to test the pharmacological responses. The preparations were treated with H₂O₂ for at least 30 min for each concentration. To evaluate the preventive and treating effects of antioxidants, ascorbic acid (1 mmol/L) or *N*-(mercaptopyrionyl)-glycine (N-MPG, 10 mmol/L, a specific scavenger of the ·OH free radical) was treated 30 min before or 60 min after H₂O₂ administration, respectively. Verapamil (calcium channel blocker, 0.1 μmol/L)

or ryanodine (ryanodine receptor blocker, 0.1 μmol/L) was administered after H₂O₂ (2 mmol/L) to elucidate the role of calcium homeostasis in H₂O₂-induced PV arrhythmogenesis. Parameters of the AP were measured with 2-Hz electrical stimuli before and after drug administration in the LA or PV without spontaneous activity, which was measured after 10 min of superfusion. The AP amplitude (APA) was obtained from the resting membrane potential (RMP) or maximum diastolic potential to the peak of AP depolarization. The AP durations at a repolarization of 90%, 50%, and 20% of the APA were measured as APD₉₀, APD₅₀, and APD₂₀, respec-

tively. The maximum upstroke velocity was acquired using the maximum positive value of the first derivative of the AP. Spontaneous activity was defined as the constant occurrence of spontaneous APs in the absence of any electrical stimuli. Burst firing was defined as the occurrence of accelerated spontaneous potential (faster than the basal rate) with sudden onset and termination.²⁷

Statistical Analysis

All continuous variables are expressed as mean±SEM. A repeated-measures ANOVA with a post-hoc LSD, or paired t-test was used to compare differences before and after drug administration. Unpaired t-test was used to compare the effects of H₂O₂ in the absence and presence of ascorbic acid or N-MPG, and the differences between PVs and LA. P<0.05 was considered statistically significant.

Results

Effects of H₂O₂ on Electrical Activity of PVs and LA

PV spontaneous activity (n=35, 73%) was found in 48 rabbit PV tissue preparations with a mean frequency of 2.0±0.1 Hz (cycle length, 503±19 ms). The PVs with spontaneous activity had smaller APA (101±2 mV vs 109±2 mV, P<0.001) and shorter APD₉₀ (89±3 ms vs 101±3 ms, P<0.05) than the PVs without spontaneous activity (n=13). The maximum diastolic potential in the PVs with spontaneous activity was less negative than the RMP in the PVs without spontaneous activity

(−74.3±0.4 mV vs −79.4±1.4 mV, P<0.001). **Figure 1A** shows examples of the effects of H₂O₂ (0.02, 0.2, and 2 mmol/L) on the PV spontaneous activity. Compared to those of the control, H₂O₂ (0.02, and 0.2 mmol/L) reduced the PV spontaneous activity by 22±5% and 17±5%, respectively. In contrast, H₂O₂ (2 mmol/L) significantly increased PV spontaneous activity by 36±10%. Moreover, H₂O₂ (0.02, 0.2, and 2 mmol/L) induced the occurrence of PV burst firing (30%, 60%, and 100%) and early afterdepolarizations (0%, 10%, and 50%) in a dose-dependent manner (**Figure 1B**). H₂O₂ at 2 mmol/L induced a higher incidence of PV burst firing and early afterdepolarizations than those at baseline or after the administration of H₂O₂ at 0.02 mmol/L.

In PVs without spontaneous activity (**Figure 2**), H₂O₂ (0.02, 0.2, and 2 mmol/L) concentration-dependently shortened the APD₉₀, APD₅₀ and APD₂₀, but H₂O₂ did not change the RMP or APA. H₂O₂ at 2 mmol/L initially prolonged the AP duration, and then shortened the AP duration during the initial administration of 15 min. Compared to those in the control, H₂O₂ (2 mmol/L) significantly increased PV contractility. Moreover, as in the examples shown in **Figure 2C**, H₂O₂ (0.02, 0.2, and 2 mmol/L) easily induced the occurrence of PV burst firing (75%, 87%, and 100%) and early afterdepolarizations (38%, 38%, and 62%). H₂O₂ (2 mmol/L) administration also produced a longer PV burst firing (13±3 s vs 1±1 s, P<0.005) than did H₂O₂ at 0.02 mmol/L.

In LA tissue (**Figure 3**), H₂O₂ (0.02, 0.2, and 2 mmol/L) concentration-dependently shortened the APD₉₀, APD₅₀

Figure 5. Preventive effects of ascorbic acid and *N*-mercaptopropionyl-glycine (N-MPG) on H₂O₂-induced pulmonary vein (PV) arrhythmogenesis. **(A)** Average data before and after administration of ascorbic acid and different concentrations (0.02, 0.2, and 2 mmol/L) of H₂O₂ on PV spontaneous activity (n=9). *P<0.05 vs baseline. #P<0.05, ##P<0.01 vs ascorbic acid. **(B)** Average data before and after administration of N-MPG and different concentrations (0.02, 0.2, and 2 mmol/L) of H₂O₂ on the PV spontaneous activity (n=6). **P<0.01 vs baseline. #P<0.05 vs N-MPG.

and APD₂₀. Similar to those in PVs, H₂O₂ at 2 mmol/L initially prolonged the AP duration, and then shortened the AP duration. Moreover, compared to those in the control, H₂O₂ (2 mmol/L) significantly increased LA contractility. Different from that in PVs without spontaneous activity, however, H₂O₂ (0.02, 0.2, and 2 mmol/L) did not induce burst firing, but occasionally induced triggered beats (10%, 10%, and 60%) or early afterdepolarizations (0%, 10%, and 10%). Moreover, H₂O₂ (0.2, and 2 mmol/L) shortened the APD₉₀ (42±3% vs 20±3%, P<0.005; and 45±3% vs 23±3%, P<0.005) or APD₅₀ (51±4% vs 26±4%, P<0.005; and 58±3% vs 32±6%,

P<0.005) in the LA to a greater extent than in the PVs. In addition, H₂O₂ (2 mmol/L) increased the contractile force in the LA to a greater extent as compared to that in the PVs without spontaneous activity (101±22% vs 42±6%, P<0.05). In order to study whether enhanced transient outward currents might contribute to the H₂O₂-induced AP duration shortening, we administered a blocker of transient outward currents (4-aminopyridine, 2 mmol/L) in the LA and found that 4-aminopyridine can reverse the H₂O₂-induced shortening of APD₉₀, APD₅₀, and APD₂₀ (Figure 4).

Figure 6. Effects of ascorbic acid and *N*-mercaptopyropionyl-glycine (N-MPG) on H_2O_2 -induced pulmonary vein (PV) arrhythmogenesis. (A) Ascorbic acid and (B) N-MPG abolished H_2O_2 -induced PV spontaneous activity. These effects were reversible after washout of ascorbic acid or N-MPG (Lower panel).

Figure 7. Effects of verapamil or ryanodine on H_2O_2 -induced pulmonary vein (PV) spontaneous activity. Verapamil (0.1 μ mol/L, n=6) or ryanodine (0.1 μ mol/L, n=6) decreased H_2O_2 -induced PV spontaneous activity. *P < 0.05, **P < 0.01, ***P < 0.005 vs baseline.

Figure 8. (A) Preventive effects of ascorbic acid on H₂O₂-induced changes of action potential (AP) morphology and contractility in pulmonary veins (PVs) without spontaneous activity. (B) PV without spontaneous activity (n=5) before and after administration of ascorbic acid and effect of different concentrations (0.02, 0.2, and 2 mmol/L) of H₂O₂. *P<0.05 vs baseline. #P<0.05 vs ascorbic acid alone. APA, AP amplitude; APD_{20,50,90}, AP duration at a repolarization of 20%, 50%, 90% of the APA; RMP, resting membrane potential.

Effects of Ascorbic Acid and N-MPG on H₂O₂-Induced PV and LA Arrhythmogenesis

Figure 5A shows the effects of ascorbic acid on PV spontaneous activity. Ascorbic acid (1 mmol/L) mildly decreased the PV spontaneous activity from 2.3 ± 0.2 Hz to 2.1 ± 0.1 Hz (n=9, P<0.05). In the presence of ascorbic acid (1 mmol/L), however, H₂O₂ (0.02, 0.2, and 2 mmol/L) decreased the PV spontaneous activity by $9 \pm 4\%$, $8 \pm 4\%$ and $13 \pm 4\%$, respectively, and that effect was reversed after washout. The effects of H₂O₂ (0.02, and 2 mmol/L) on PV spontaneous activity significantly differed between those in the presence and absence of ascorbic acid. In the presence of ascorbic acid (1 mmol/L), H₂O₂ (0.02, 0.2, and 2 mmol/L) occasionally

induced PV burst firing (0%, 33%, and 33%) and early after-depolarizations (0%, 0%, and 11%). Compared to that without ascorbic acid, H₂O₂ (2 mmol/L) in the presence of ascorbic acid produced a lower incidence of PV burst firing (33% vs 100%, P<0.01).

N-MPG (10 mmol/L) decreased the PV spontaneous activity from 2.0 ± 0.1 Hz to 1.3 ± 0.1 Hz (n=6, P<0.05; Figure 5B). In the presence of N-MPG (10 mmol/L), however, H₂O₂ (0.02, 0.2, and 2 mmol/L) decreased PV spontaneous activity by $16 \pm 4\%$, $16 \pm 5\%$, $19 \pm 8\%$, respectively, in a non-concentration dependent manner, and this effect was reversed after washout. Similar to that in the presence of ascorbic acid, in the presence of N-MPG, H₂O₂ (0.02, 0.2, and 2 mmol/L)

occasionally induced PV burst firing (17%, 17%, and 33%) and early afterdepolarizations (0%, 16%, and 16%). Compared to that in the absence of N-MPG, H₂O₂ (2 mmol/L) in the presence of N-MPG produced a lower incidence of PV burst firing.

Figure 6 shows examples of the effects of ascorbic acid (1 mmol/L) and N-MPG (10 mmol/L) on H₂O₂ (2 mmol/L)-induced PV spontaneous activity. Ascorbic acid (1 mmol/L) reduced the H₂O₂-induced PV spontaneous activity from 3.7 ± 0.3 Hz to 1.9 ± 0.1 Hz ($n=7$, $P<0.05$) and abolished the occurrence of PV burst firing. Similarly, N-MPG (10 mmol/L) also reduced H₂O₂-induced PV spontaneous activity from 3.6 ± 0.1 Hz to 1.3 ± 0.3 Hz ($n=5$, $P<0.05$), and abolished PV burst firing. Both of those effects were reversible after washout (**Figure 6**). As the example shown in **Figure 7A**, verapamil (0.1 μ mol/L) significantly decreased the H₂O₂ (2 mmol/L)-induced PV spontaneous activity. Similarly, ryanodine (0.1 μ mol/L) also reduced H₂O₂ (2 mmol/L)-induced PV spontaneous activity (**Figure 7B**).

In PVs without spontaneous activity, ascorbic acid (1 mmol/L) shortened the APD₉₀, and APD₅₀, but not the APD₂₀, RMP, APA or contractility (**Figure 8**). In the presence of ascorbic acid (1 mmol/L), however, H₂O₂ shortened only the APD₅₀ and reduced contractility at a concentration of 2 mmol/L. H₂O₂ (0.02, 0.2, and 2 mmol/L) had no significant effects on the APD₂₀, RMP, APA, or APD₉₀.

Discussion

Oxidative stress plays a vital role in the pathophysiology of a wide range of cardiovascular diseases including AF.^{7–10,28} Oxidative stress may mediate the pathological process of heart failure, inflammation, ischemia, and renin–angiotensin or sympathetic–adrenergic activations,²⁸ which are known to enhance the occurrence of AF. In the present study we found that H₂O₂ at 2 mmol/L significantly increased the rate of PV ectopic beats. In addition, H₂O₂ concentration-dependently induced or prolonged the occurrence of PV burst firing. Because the electrophysiological characteristics of PV burst firing are similar to those in clinical focal AF or atrial tachycardia, these findings strongly suggest that oxidative stress has a direct arrhythmogenic effect on PVs. Previous studies showed that oxidative stress can induce Ca²⁺ overload by changing the Ca²⁺ regulatory proteins or cell membrane lipid oxidation status.²⁸ In addition, H₂O₂ also enhances Ca²⁺ release through increasing the opening probability of ryanodine receptors.²⁹ Taken together, these effects may contribute to the arrhythmogenic activity of H₂O₂. Because PVs have abnormal Ca²⁺ homeostasis and a less negative RMP,^{20,21,30–33} the increase in intracellular Ca²⁺ and Ca²⁺ release could easily induce the occurrence of PV burst firing and genesis of early afterdepolarizations. The present results show that H₂O₂-induced PV spontaneous activity could be reduced by verapamil and ryanodine. These findings collectively imply that intracellular Ca²⁺ overload may contribute to H₂O₂-induced PV arrhythmogenesis and also indicate a high susceptibility to oxidative stress in PVs as compared to the LA. Taken together, it is reasonable to assume that oxidative stress has a high arrhythmogenic potential for inducing AF by enhancing PV electrical activity. Previous studies, however, have indicated that clinically relevant ranges of H₂O₂ were expected to have a magnitude of μ mol/L. Therefore, the concentration (2 mmol/L) of H₂O₂ used in the present study may be supra-physiological. Additionally, the increases in intracellular Ca²⁺ and Ca²⁺ release by H₂O₂ may also enhance the

contractility of PVs and LA as we found. Because PV cardiomyocytes contain pacemaker currents,^{20–22} the known inhibitory effects of H₂O₂ on the pacemaker currents may contribute to a slowing of the PV spontaneous activity during the administration of low concentrations (0.2, and 0.02 mmol/L) of H₂O₂.³⁴

Differing from that in ventricular myocytes,³⁵ H₂O₂ can shorten the AP duration in PVs and LA. Because transient outward currents contribute more to the AP duration in PVs and LA cardiomyocytes than in ventricular myocytes, the shortening of AP duration by H₂O₂ was proposed to arise from its known effect on increasing transient outward currents.³⁶ The present finding that 4-aminopyridine could reverse the H₂O₂-induced shortening of AP duration in LA also suggests this mechanism. Moreover, H₂O₂ shortened the AP duration to a greater extent in LA than in PVs. This effect can increase dispersion of AP duration to enhance the genesis of reentry circuit. Additionally, oxidative stress may induce anatomical changes and atrial fibrosis,³⁷ which can decrease conduction velocity and contribute to the maintenance of AF.³⁸

Antioxidant agents have been shown to have an antiarrhythmic potential. A previous study showed that ascorbic acid can reduce the occurrence of postoperative AF and attenuate the electrical remodeling from rapid atrial pacing.¹³ It is not clear, however, whether antioxidant agents can reduce the occurrence of AF though decreasing PV arrhythmogenesis. In the present study we found that ascorbic acid has direct electrophysiological effects on PVs with the decrease of spontaneous activity and shortening of APD₉₀ and APD₅₀. Moreover, ascorbic acid and N-MPG significantly attenuated the arrhythmogenic effects of H₂O₂. Through the scavenging of reactive oxidative stresses to ameliorate the Ca²⁺ overload, both ascorbic acid and N-MPG reduced H₂O₂-induced PV burst firing and genesis of early afterdepolarizations. Therefore, antioxidant agents are effective in preventing the arrhythmogenesis induced by oxidative stress in PVs. Furthermore, during H₂O₂-induced PV arrhythmogenesis, the administration of N-MPG or ascorbic acid not only reduced the PV spontaneous activity but also abolished the PV burst firing. These results indicate that acute administration of antioxidant agents can reverse arrhythmogenesis due to oxidative stress. Therefore, antioxidant agents should be a promising antiarrhythmogenic therapy in AF enhanced by oxidative stress such as heart failure, sepsis or activation of the renin–angiotensin, and adrenergic systems. Previous reports, however, have found that antioxidants such as β -carotene, vitamin C or E produced little or controversial clinical benefit,^{2–5} despite the beneficial effects of antioxidants shown in *in vitro* or animal studies in the field of atherosclerosis.⁶ The possible dissociation between *in vivo* and *in vitro* studies should be taken into consideration. Further clinical study is warranted to confirm the antioxidant effect on the prevention of AF.

Conclusions

The present study has demonstrated that H₂O₂ significantly changes the electrophysiological characteristics of PV and LA through activation of free radicals and may facilitate the occurrence of AF.

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