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Protective Effects of Apocynin on Atrial Electrical Remodeling and Oxidative Stress in a Rabbit Rapid Atrial Pacing Model

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Abstract

It has been proposed that apocynin might be used in the prevention and management of atrial fibrillation (AF). The purpose of this study was to investigate the effects of apocynin on atrial electrical remodeling and oxidative stress promoted by rapid atrial pacing (RAP) in rabbits. New Zealand white rabbits were subjected to RAP with or without apocynin treatment. Serial electrophysiological studies (EPS) were performed at baseline and every half hour after RAP onset. Superoxide dismutase (SOD) and lactate dehydrogenase (LDH) activities and Ca^{2+} content in tissue homogenates of both atria were assayed after EPS. In the RAP group but not in the sham-operated and RAP with apocynin groups, atrial effective refractory periods (AERPs) at cycle length of 200 and 150 ms shortened most clearly by 20.8 $\pm$ 10.2 ms at 3 h ($P < 0.001$) and by 12.8 $\pm$ 11.1 ms at 2 h ($P < 0.05$) respectively, and AERP rate adaptation decreased to minus values. Higher AF inducibility (66.7%) and longer AF duration (an average of 37.8 min) were presented in the RAP group. Compared with the other groups, SOD activity was lower, and LDH activity and Ca^{2+} content were higher in the RAP group. Similar differences were not found between the sham-operated and the RAP with apocynin treatment groups. These data show that apocynin attenuates the development of atrial electrical remodeling in a short period of 3-h RAP, and reduces RAP-mediated inducibility and duration of AF in this model.

Key Words: apocynin, atrial fibrillation, oxidative stress

Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia encountered in clinical practice. In Chinese adults, the age-adjusted prevalence of AF is 0.74% in men and 0.72% in women, and the prevalence increases with age (16). Pathophysiology of AF remains not well established despite extensive studies. However, compelling evidences point to atrial structural and electrical remodeling as critical in AF pathophysiology (18).

Oxidative stress results from an imbalance between the formation of free radicals and their scavengers in the tissue, and causes accumulation of reactive oxygen species (ROS) in the tissue (20). Recent studies have found that oxidative stress, which may reinforce the effect on the pathogenesis of AF, has a major impact on atrial structural and electrical remodeling with increased NADPH oxidase activity potentially playing a key role in the initiation of this damage course (5, 10-13).

Apocynin is a naturally-occurring organic compound isolated from a variety of plants and is structurally related to vanillin. It is a potent inhibitor of NADPH oxidase (26). Apocynin has been shown to suppress NADPH oxidase activity and to lower nitric

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oxide and hydrogen peroxide production in vascular smooth muscle cells without causing any noticeable side effects in rats (34, 35). In angiotensin II-infused mice, oral apocynin prevented increase of systolic blood pressure, improved acetylcholine-mediated vasodilation, and inhibited the increases in vascular NADPH oxidase activities (30). It was proposed that apocynin might be used in the prevention and management of AF (25). The purpose of the current study is to evaluate the protective effects of apocynin on electrical remodeling and atrial oxidative stress induced by short period of rapid atrial pacing (RAP) which imitates the forepart of paroxysmal AF in rabbits.

Materials and Methods

Animal Model

The principles of laboratory animal care (NIH publication No. 8623, revised 1985) were followed. Eighteen New Zealand white rabbits (2.5 ± 0.2 kg) of either gender were provided by Medical Experimental Animal Center of Chinese PLA General Hospital. All the rabbits were confirmed to be healthy on history and physical examination. Rabbits were anesthetized with 20% urethane (1 g/kg i.v.) solution in the supine position. A 5 F electrode (quadripolar, interelectrode distance 1 cm) was inserted through the jugular vein into the right atrium (RA) for stimulation and recording during electrophysiological studies (EPS). The pulse width was 2 microsecond (ms), and the output voltage was set to double pacing threshold. The pacemaker was programmed to maximal atrial capture rate (500 to 600 bpm) during RAP. Baseline EPS was performed after i.v. injection of metoprolol tartrate (200 μg/kg, followed by 20 μg/kg/h) and atropine sulfate (40 μg/kg, followed by 7 μg/kg/h) to block the cardiac effect of autonomic nerves. RAP was then initiated, and EPS was repeated every 0.5 h for 3 h.

Groups

Eighteen rabbits were randomly divided into three groups. Six rabbits in the sham-operated group were administered normal saline 3 ml/kg/d orally beginning 3 days before EPS without RAP. The other twelve rabbits were subjected to RAP, oral pre-administration with 1) normal saline 3 ml/kg/d (n = 6, RAP group); or 2) apocynin (purity 98%) 15 mg/d (n = 6, APO+RAP group) at a concentration of 10 mg/ml, beginning 3 days before RAP onset (21). Tween 80 (0.05%) was used to solubilize the apocynin powder in an appropriate amount of sterile water. The mixture was stored in the dark at 4°C, and was shaken sufficiently before use.

Experimental Procedure In Vivo

Right atrial effective refractory period (AERP) was measured at basic cycle lengths (BCLs) of 200 ($AERP_{200}$) and 150 ($AERP_{150}$) ms with 8 basic stimuli (S_1) followed by a premature extrastimulus (S_2) with 2-ms increments. The delay of stimulation was 5 s. The shortest S_1-S_2 interval resulting in a propagated atrial response was taken as the AERP (24). The average value of three repeated tests was taken as $AERP_{200}$ or $AERP_{150}$. The total measurement time was not over 5 min. ($AERP_{200} - AERP_{150}$)/50 ms defined the formula of AERP rate adaptation. AF inducibility was determined as the percentage of atrial irregular rapid electrical activity recorded with an intracardiac electrode sustaining for over 1 s induced by RAP. Mean AF duration was calculated in each group of rabbits which suffered from RAP-induced AF.

Experimental Introduction In Vitro

After EPS, tissue samples of RA and the left atrium (LA) were fast-frozen and stored at -80°C for further tests. Tissue homogenates with different specific concentrations were prepared for the following spectrophotometer-based tests. Protein content in the tissue homogenate was determined with the Coomassie Brilliant Blue method. Then the optical density (OD) of each reaction was measured in specific wavelength, and the parameters of every unit of protein were calculated according to the formula provided by the kit. Superoxide dismutase (SOD) activity, in active unit per mg of protein (U/mg prot), was quantified by a xanthine oxidase assay. The xanthine oxidase reaction system produces superoxide anion which oxidizes hydroxylamine to nitrite showing purple with chromogenic reagent. OD was measured at 550 nm wavelength. Therefore, U/mg prot used as the unit of SOD activity is defined as the SOD capacity for inhibiting 50% of the absorbance per mg tissue protein in 1 ml reaction solution. Lactate dehydrogenase (LDH) catalyzes lactic acid to produce pyruvic acid. Two nitrobenzene hydrazone pyruvic acid, produced by reaction of pyruvic acid and 2,4-dinitrophenylhydrazine, is brown in alkaline solution. Hence, LDH activity, in unit of U/mg prot, was calculated after OD values measurements at 440 nm wavelength. And 1 U/mg prot of LDH activity defines the capacity for producing 1 μmol pyruvic acid after reacting in a specified condition (with lactic acid buffer at 37°C for 15 min) corresponding to 1 mg tissue protein. In alkaline solution, Ca^{2+} can combine with methyl thymol blue to form blue complexes, and OD values are measured at 610 nm wavelength. Ca^{2+} content, in unit of millimole per gram protein (mmol/g prot), was quantified by methyl thymol blue

Table 1. Mean $AERP_{200}$ values in RA during serial EPS

	0 h	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h
Sham-operated	94.0 ± 5.5	93.8 ± 7.1	92.8 ± 7.3	91.2 ± 6.7	94.2 ± 7.2	92.8 ± 6.1	90.3 ± 7.4
RAP	93.0 ± 6.2	86.8 ± 5.5	81.3 ± 4.9*	79.6 ± 3.4†	74.6 ± 7.0†	76.1 ± 8.0†	71.5 ± 5.8†
APO+RAP	96.8 ± 6.6	96.7 ± 7.4	96.0 ± 3.4	94.7 ± 3.4	93.5 ± 6.9	94.5 ± 8.2	94.7 ± 9.1

(n = 5~6, mean ± SD, ms) *P < 0.01, $^{\dagger}P$ < 0.001, compared with 0 h in the same group. n = 5, only from 1 h to 3 h in RAP group.

Table 2. Mean $AERP_{150}$ values in RA during serial EPS

	0 h	0.5 h	1 h	1.5 h	2 h	2.5 h	3 h
Sham-operated	90.2 ± 7.6	92.0 ± 9.4	88.8 ± 7.7	89.2 ± 8.3	88.7 ± 6.8	87.3 ± 7.1	88.2 ± 7.0
RAP	89.1 ± 4.8	83.5 ± 6.6	79.6 ± 8.4	79.6 ± 10.5	75.6 ± 10.4*	76.3 ± 12.9*	77.3 ± 10.0†
APO+RAP	91.8 ± 8.8	92.5 ± 6.2	92.7 ± 3.9	91.5 ± 7.5	91.2 ± 6.4	94.0 ± 9.4	93.3 ± 7.7

(n = 5~6, mean ± SD, ms) *P < 0.05, $^{\dagger}P$ = 0.055, compared with 0 h in the same group. n = 5, only from 1 h to 3 h in RAP group.

colorimetric method.

Statistical Analysis

Statistical analysis was performed with SPSS version 17.0. Data are presented as means ± standard deviation (SD). Analyses of variance (ANOVA) were performed for analyzing electrophysiological data (including $AERP_{200}$, $AERP_{150}$, and AERP rate adaptation) in each group, followed by a Dunnett *t*-test (2-tailed) to compare each result with the baseline in the same group. Comparisons of the baseline $AERP_{200}$ and $AERP_{150}$ between the three groups were analyzed by ANOVA followed by a Bonferroni-corrected *t*-test (2-tailed). *Chi*-square tests were performed for comparing AF inducibility between RAP and APO+RAP groups. Oxidative stress parameters of multiple-group comparisons were analyzed by a Bonferroni-corrected *t*-test (2-tailed) following ANOVA. Comparisons of oxidative stress parameters between LA and RA in the same group were analyzed by Paired Sample *t*-test. A 2-tailed *P*-value < 0.05 was considered statistically significant.

Results

Effects of Apocynin on AERP

The changes of $AERP_{200}$ and $AERP_{150}$ in rabbits are shown in Tables 1 and 2. Under baseline conditions (0 h), there was no significant difference in $AERP_{200}$ and $AERP_{150}$ among the three groups (P = 0.132 and P = 0.760, respectively). In the RAP group, $AERP_{200}$ and $AERP_{150}$ decreased substantially along with prolongation of RAP, shortened most clearly by 20.8 ± 10.2 ms at 3 h (P < 0.001) and 12.8 ± 11.1 ms at 2 h (P < 0.05), respectively. In contrast, AERP in the sham-operated and APO+RAP groups showed no significant changes during serial EPS.

Effects of Apocynin on AERP Rate Adaptation

The AERP rate adaptation of the rabbits in the sham-operated group did not change significantly during the study (P = 0.369). In the RAP group, the AERP rate adaptation of the rabbits decreased gradually, and reversed to -0.11 ± 0.18 (*vs*. 0.08 ± 0.08 under baseline conditions, P < 0.05) at the end of EPS. In the APO+RAP group, the loss of AERP rate adaptation (0.03 ± 0.05 at the end of EPS *vs*. 0.14 ± 0.11 under baseline conditions, P = 0.065) indicated no significance.

Effects of Apocynin on Inducibility and Duration of AF

In the sham-operated group, no AF was detected during EPS. In the RAP group, four rabbits suffered from induced AF which lasted for 1, 5, 25 and 120 min, respectively. AF inducibility was 66.7% and the mean AF duration was 37.8 min. In the APO+RAP group, only one rabbit suffered from RAP-induced AF which lasted for 4 min, and AF inducibility was 16.7% (compared with RAP group, P = 0.079).

Effects of Apocynin on SOD and LDH Activities

The comparison of SOD and LDH activities among the three groups is shown in Table 3. In the

Table 3. Oxidative stress parameters compared among three groups

	SOD activity (U/mg prot)		LDH activity (U/mg prot)		Ca^{2+} content (mmol/g prot)	
	LA	RA	LA	RA	LA	RA
Sham-operated	177.6 ± 6.2	169.5 ± 13.1	46.8 ± 4.6	42.7 ± 3.0	0.09 ± 0.02	0.07 ± 0.02
RAP	131.1 ± 15.6†	142.8 ± 12.9*	57.4 ± 2.4*	47.6 ± 2.0*	0.18 ± 0.04*	0.26 ± 0.11*
APO+RAP	154.8 ± 9.2‡	171.2 ± 12.8‡	48.8 ± 6.9	41.3 ± 2.6§	0.16 ± 0.05	0.17 ± 0.08

n = 6, mean ± SD is shown. *$P < 0.05$, $^{\dagger}P < 0.01$, compared with the sham-operated group in the same atrium; $^{\ddagger}P < 0.05$, $^{\S}P < 0.01$, compared with the RAP group in the same atrium.

RAP group, when compared with the sham-operated group in both LA and RA, the SOD activity decreased significantly ($P < 0.01$ and $P < 0.05$, respectively) and the LDH activity increased significantly ($P < 0.05$). In the APO+RAP group, the SOD activity increased significantly ($P < 0.05$ in both atria) and the LDH activity decreased significantly (in LA $P = 0.056$ and in RA $P < 0.01$) compared with the RAP group in both atria. SOD activities decreased in LA compared with the sham-operated group ($P = 0.052$). Comparison within the same group showed that there was no apparent difference of SOD activities between LA and RA in each group, whereas LDH activity in LA was higher than RA in the RAP ($P < 0.01$) and APO+RAP ($P = 0.054$) groups.

Effects of Apocynin on Ca^{2+} Content

The comparison of Ca^{2+} content among the three groups is shown in Table 3. Compared with the sham-operated group, the Ca^{2+} content in both LA and RA ($P < 0.05$) increased significantly in the RAP group. Although the Ca^{2+} content in the APO+RAP group was higher than that of the sham-operated group and lower than that of the RAP group in both atria, the differences were not statistical significance. There was no significant difference between LA and RA compared within each group.

Discussion

Experiments on animals and human studies have found that increases in NADPH oxidase activities play a key role in the generation of ROS in atrial tissues and in AF (5, 11). Most studies indicated that apocynin was a potent inhibitor of NADPH oxidase (25, 26, 30, 34, 35). There was, however, one study that considered apocynin functioned in endothelial cells and vascular smooth muscle cells mainly as an antioxidant (9). Although it is hypothesized that apocynin could be effective against AF, to the best of our knowledge, no direct evidence has been presented to demonstrate that apocynin can prevent atrial electrical remodeling *in vivo*. In this study, it is demonstrated that RAP leads to both acute atrial electrical remodeling and oxidative stress injury in rabbits. Apocynin attenuates atrial electrical remodeling and oxidative stress in atria induced by RAP in rabbits.

Potential Role of Ca^{2+} Handling in Atrial Electrical Remodeling

Previous studies have demonstrated that after the onset of AF or RAP, AERP shortened and physiological effective refractory period (ERP) rate adaptation was reduced (31, 33). The same results are observed in our study that in the RAP group, $AERP_{200}$ and $AERP_{150}$ decreased and the AERP rate adaptation was reversed. Moreover, AF inducibility was higher and mean AF duration was longer in the RAP group, and were significantly different from the sham-operated group. As demonstrated in this study, atrial 'electrical remodeling' produces a substrate favorable for AF. The signal transduction leading to AERP abbreviation in AF is still unclear. Altered intracellular Ca^{2+} handling is believed to play an important role (19, 27, 33). It has been shown previously that RAP and AF increase the intracellular Ca^{2+}-overload in atrial myocytes (7), which induces complex changes in intracellular Ca^{2+} handling (33). Tachycardia increases Ca^{2+} loading directly by increasing the frequency of action potentials during which depolarization-induced Ca^{2+} current carries Ca^{2+} into the cell (27). Our study presents similarly that RAP induces increase of Ca^{2+} content in both atrial tissue homogenates compared with sham-operated group. The Ca^{2+} overloading owing to increased rate is prevented by short- and long-term adaptations that reduce Ca^{2+} entry at the expense of atrial action potential duration (APD) abbreviation, positively feeding back on AF likelihood by reducing ERP and wavelength (19). Our study partially verified this hypothesis. The electrophysiological changes, for instance decreases of $AERP_{200}$, $AERP_{150}$ and AERP rate adaptation, are consistent with increase of total Ca^{2+} content in atrial tissue in the RAP group.

The Link between Ca^{2+} Accumulation and Oxidative Stress

It has been suggested that atrial tachycardia-

induced Ca^{2+} accumulation leads to increased oxidative stress, and, thus, the resulting changes in the cellular redox state facilitate the genesis and perpetuation of atrial arrhythmias (3, 29). Furthermore, a previous study has found that pacing of atrial myocytes increases the intracellular ROS levels (28). Even under steady-state conditions, the presence of the products of ROS interactions with macromolecules activates antioxidative defenses such as SOD (14). On the other hand, it is shown that oxidative stress can also induce Ca^{2+}-overload by changing the status of Ca^{2+} regulatory proteins or cell membrane lipid oxidation (4). As is similarly showed in our study that RAP for 3 h induced a decrease of SOD activity and an increase of LDH activity that are consistent with increases of the Ca^{2+} content in atrial tissue homogenates in rabbits. The decrease of SOD activity indicates that ROS scavenging capacity is reduced in both atria. A previous study considered an increase in SOD activity to have a protective effect by decreasing oxidative stress (2). These changes demonstrate that the redox equilibrium in the atrial tissue is broken, which may facilitate the increase of inducibility and duration of AF in the RAP group. Because three parameters were measured after EPS at the same time, the causality regarding calcium accumulation and oxidative stress in atrial myocytes during rapid pacing is still unknown.

Possible Mechanism of Apocynin Prevention of Atrial Electrical Remodeling

It is well known that NADPH oxidase is a major component of various ROS-producing oxidation systems that can cause oxidative stress and mediate occurrence and development of AF (6). Previous studies have strongly suggested that tachycardia increases the generation of ROS through NADPH oxidase activation (23).

Of note, apocynin is a well-known membrane NADPH oxidase inhibitor. And it had no effect on the ventricular ERP in rabbits in the sham group (15), which was similar that in our study comparing between all the three groups at the baseline apocynin was not found to prolong AERPs or to increase AERP rate adaptation without RAP. Apocynin attenuated rapid electrical field stimulation-induced intracellular ROS generation in atrial myocytes, which suggests that this process is membrane NAPDH oxidase-dependent (28). In the presence of RAP in this study, apocynin prevented atrial 'electrical remodeling' effectively and decreased the inducibility and duration of AF. Apocynin was confirmed to correlate with decreased vascular ROS and NOx levels in the aortic tissues from NADPH oxidase inhibition in a murine model (1, 32). Analogously, apocynin attenuated the decrease of SOD activity and the increase of LDH activity in our research, which suggests that apocynin reduces RAP-induced oxidative stress by inhibiting NADPH oxidase. The mechanism of apocynin inhibiting NADPH oxidase has been demonstrated in endothelial cells and neutrophils using immunoblots of cell membranes, where apocynin has been shown to inhibit the translocation of cytosolic oxidase subunits to the membrane, thus preventing the assembly and activation of a functional NADPH oxidase complex (8, 17). Furthermore, there is evidence to demonstrate that the NADPH oxidase inhibitor apocynin could reduce Ca^{2+} release activity (22, 23). In our study, apocynin mitigates Ca^{2+} accumulation resulting from RAP in rabbit atrial tissues. As has been noted, attenuating oxidative stress and Ca^{2+} accumulation by means of inhibiting NADPH oxidase may be the underlining mechanism of apocynin preventing acute atrial electrical remodeling promoted by RAP.

Limitations

Firstly, NADPH oxidase (especially its competent subunits) was not quantified directly in our study. SOD and LDH activities were used to reflect both the degree of oxidative stress and the effect of NADPH oxidase. It is quite possible that other markers of oxidative stress may provide additional information on the effect of apocynin on preventing acute atrial electrical remodeling. Secondly, apocynin preventing Ca^{2+} accumulation *via* inhibiting NADPH oxidase was only indirectly proved. Pretreatment with apocynin 3 days before RAP was shown to mitigate the Ca^{2+} content in tissue homogenates of both atria. Thirdly, the oxidative stress parameters of both atria were compared between LA and RA. Tissues from pulmonary veins (PVs) were mixed with muscle and vascular cells. The oxidative stress parameters in PVs could not be measured using the methods of the present study. Fourthly, the effect of apocynin in some other mechanisms that may contribute to AERP shortening in early stage of rapid activation could not be denied in our study, for instance block of ion channel or calcium influx. Fifthly, in our study apocynin was proved to be effective in preventing acute changes in AERP. However, whether apocynin is successful in preventing electrophysiologic changes over a longer term of atrial pacing remains unknown. Further studies are needed to resolve the above limitations.

In conclusion, this study presents data to indicate that apocynin is able to attenuate the development of acute atrial electrical remodeling and RAP-induced AF inducibility and duration in 3-h RAP model in rabbits. Inhibition of oxidative stress could be the

underlying mechanism for apocynin-mediated prevention of AF development.

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