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Effects of Resveratrol on Oxidative Stress Injury Induced by Rapid-Pacing in Isolated Rabbit Hearts

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Abstract

Oxidative stress injury plays an important role in the process of atrial remodeling. The mechanisms of oxidative stress injury in atrial by rapid pacing and the protective effects of resveratrol will be explored in this study.

Thirty-two isolated rabbit hearts were produced by rapid atrial pacing. They were randomly divided into 4 groups: control group (Ctrl group), Rapid Atrial Pacing group (RAP group), Apocynin Pretreatment group (APO group) and Resveratrol Pretreatment group (RES group); each with 8 rabbits. At the end, the indexs of the oxdise stress were measured take advantage of the techniques of the immunohistochemistry, western blotting and reverse transcription PCR.

Keywords: Oxidative stress; NADPH oxidase; Resveratrol; Rapid pacing; Isolated rabbits

Introduction

Atrial Fibrillation (AF), the most common sustained arrhythmia, is characterized by atrial electrical, contractile, and structural remodeling [1,2]. Structural remodeling includes interstitial fibrosis, cellular remodeling in atrial myocytes, such as degradation/loss of myofibrils and glycogen deposition (also known as myolysis) [3]. AF itself further augments these responses to perpetuate AF [4,5]. The mechanisms underlying both the initiation and perpetuation of AF are not well established but are thought to involve inflammation and oxidative stress [6-8]. Recently oxidative stress has been linked with atrial fibrillation [9]. There is evidence for enhanced oxidative stress in atrial tissue samples from AF patients [10].

Resveratrol is present in various dietary sources, including grapes, peanuts, plums and many plants. Much recent attention was paid to the "French paradox" which provides evidence for the beneficial effects of red wine. The antioxidant capacities of a wide variety of fruits have been reported by other laboratories [11-13]. Resveratrol has been cited in recent investigations for its possible antioxidant role and protective effects against certain forms of oxidant damage.

Our primer study showed resveratrol could prevent atherosclerosis through inhibiting platelet aggregation and could inhibit the proliferation of vascular smooth muscle cells [14,15]. In the present study, we sought to investigate the antioxidant of resveratrol on the treatment of the atrial fibrillation.

Materials and Methods

Methods

Thirty-two isolated rabbit hearts were produced by rapid atrial pacing. They were randomly divided into 4 groups: control group (Ctrl group), Rapid Atrial Pacing group (RAP group), Apocynin Pretreatment group (APO group) and Resveratrol Pretreatment group (RES group); each with 8 rabbits. At the end, the indexes of the oxidase stress were measured take advantage of the techniques of the immunohistochemistry, western blotting and reverse transcription PCR.

Animals and reagents

Approval for these experiments was obtained in advance from the Animal Ethics Committee of the Xuzhou Medical College. Rabbits (Xuzhou, Medical College, China) weighing 2.5 ± 0.3 kg were randomly divided into 4 groups: (1) Ctrl group; (2) Rapid Atrial Pacing group (RAP); (3) RAP plus apocynin (APO); (4) RAP plus resveratrol (RES). Resveratrol and apocynin (purity>99 %) were purchased from Sigma Aldrich (Fluka, Germany), and dissolved in dimethylsulfoxide. Primary antibody cTnT (ab10218) was purchased from Abcam, USA; primary antibody Anti-NOX2/gp91phox (bs-3889R) was purchased from Bioss, Beijing, China; and anti-calpain1(BA0679) was purchased from BOSTER, Wuhan, China. Detection kit for Superoxide dismutase (SOD) and Maleic Dialdehyde (MDA) were purchased from Jiancheng Bioengineering Institute (Nanjing, China); and the detection kit for ROS. NADPH oxidase activities were purchased from GENMED SCIENTIFIC INC.U.S.A. The kit for RT-PCR was purchased from promega U.S.A.

Isolated rabbit heart perfusion and experimental protocol

The procedure of the isolated rabbit hearts perfusion has been described elsewhere [16]. Briefly, 32 rabbits of either sex were injected intraperitoneally with 5,000 U/kg heparin for ten min before being anesthetized with 150 mg/kg pentobarbital. The hearts were excised; the ascending aorta was cannulated and immediately retrogradely perfused using the Langendorff technique with an oxygenated Krebs-Henseleit buffer containing (mmol/L): 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose and 1.25 $CaCl_2$. The perfusion pressure was kept constant at 80 mmHg. The perfusate, bubbled with

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95 % O_2 and 5% CO_2, was adjusted to pH 7.4 and kept at 37°C. The control group was constantly perfused for 70 min; The RAP group was firstly perfused for 40 min, and then we take use of BL-420 S with stimulation of voltage 4.0 V, frequency 10 HZ to stimulate the right auricular appendage; The APO group was firstly perfused with apocynin for 40 min, and then stimulated the right auricular for 30 min; The RES group was firstly perfused with resveratrol for 40 min, and then stimulated the right auricular for 30 min.

After perfusion, the small portions of the right and left atrial tissues were rapidly excised for histological and biochemical analyses. The histology of the atrial tissue was evaluated by haematoxylin and eosin (HE) staining.

Evaluation of the SOD and MDA in the serum of the right atrial

The levels of SOD and MDA in the right atrial of rabbits were detected by colorimetric method of xanthine oxidase and thiobarbituric acid with kits (Nanjing jian cheng Bioengineering Institute, Nanjing, China) respectively. The absorbance was measured with UV–Vis spectrophotometer (Thermo, Japan) at the wave-length of 550 nm and 532 nm respectively, according to the instructions of the kits.

Measurement of ROS level and NADPH oxidase activity in the right atrial

ROS levels in atrial tissues were detected by GENMED Tissue Superoxide Anion Colorimetric Quantitative Determination kit (GENMED). These procedures were performed according to the manufacturer's instructions.

NADPH oxidase activity in atrial tissues was detected by GENMED Tissue NADPH oxidase activity colorimetric quantitative determination kit (GENMED). These procedures were performed according to the manufacturer's instructions.

Western blot analysis

Protein expression was examined by western blot analysis as previously described [17]. Briefly, after protein concentration quantitation with the modified Bradford assay, equivalent amounts (40 μgm) of protein samples were loaded and separated by 8-12 % sodium dodecylsulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. After blocking with 5 % nonfat dry milk in Tris-buffered saline containing 0.1 % Tween 20, membranes were immunoblotted overnight at 4°c with primary antibodies including calpain1(1:400), cTnT(1:1000), gp91phox(1:500) and β-actin (1:1000) followed by incubation with the corresponding secondary antibodies at room temperature for 2 h. Protein bands were shown by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. The membranes were scanned into the computer, and the relative intensity of bands was analyzed by the Image J 3.0 system.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from frozen tissue of the right atrial by TRIzol reagent (Gibco BRL). The amount and quantity of total RNA was determined spectrophotometricslly at 260 nm and 280 nm. One microgram of total RNA was used for single strand cDNA synthesis and RNA was then converted to complementart deoxyribonucleic acid by reverse transcription with random hexa nucleotides (Promega, USA). All the alternatively spliced messenger RNA (mRNA) was amplified to yield a single DNA band on agarose gel electrophoresis. The glyceraldehyde-3-phosphate dehydrogenase gene (GADPH) was used as the house-keeping gene. Primers of the housekeeping gene and target gene were added for co-amplification in all reaction. The reaction mixture was denatured (94°c for 2 min), run for 30 cycles at 94°c denaturing for 30 seconds, annealed at 60 °c for 1 min and elongated at 68°c for 2 min, with a final extension period of 7 min at 68 °c. The reaction products were analyzed by 3% agarose gel electrophoresis. Ethidium bromide was added for optic densitometry. Reaction products were purified for DNA sequence analysis (Shenggong, ShangHai). The following primers were used: CYBA (224bp) sense 5' cagtggtacttcggcacctac 3', Anti-sense 5' aggatggtagcaaggaggaag 3'; calpain (492bp) sense 5 'cagatcatcctcaaggcactg 3', Anti-sense 5' gcgatccaggacatagacgta; gp91 (171 bp) sense 5' aaccctcctatgacctggaaat 3' , Anti-sense 5' cacctccatcttgaatcctttc; GADPH (197bp) sense 5' gcctggagaaagctgctaagt 3', Anti-sense 5' ccaggaaatgagcttcacaaa 3'.

Statistical analysis

For each experimental series, data were presented as means ± SEM. Statistical analysis was performed with Graph Pad Prism 5.0 Statistical significance (P<0.05) for each variable was estimated by ANOVA or q-test.

Result

Serum anti-oxidation analysis

To evaluate the antioxidant effect of resveratrol in our experiment, we detected the right atrial levels of SOD, ROS and MDA, which are two commonly used standards to access antioxidant ability. The results were listed in (Table 1 and 2). We can draw from the tables that, comparing to the control group, the SOD levels of rabbit right atrial in RAP group dropped greatly (P<0.01), while the MDA and ROS levels increased remarkably in the RAP group (P<0.01); To the RAP group, the levels of SOD increased and the levels of MDA and ROS reduced significantly in the RAP group and the RES group (P<0.05); But the differences of levels of SOD, ROS and MDA were no statistically significant between the APO group and the RES group (P>0.05).

And we also detect the NADPH oxidase activity. The results were as shown in (Table 2). From the table, we can draw a conclusion that, comparing to the control group, the activity of the NADPH of rabbits' right atrial in model group increased remarkably (P<0.01); To the RAP group, the activity of the NADPH oxidase reduced significantly in the APO group and the RES group (P<0.05); But the difference of the activities of the NADPH oxidase were no statistically significant between the APO group and the RES group (P>0.05).

Histopathology

Macroscopically, the differences were observed among the four groups on HE staining. The control group presented with abnormal myocardial cells (Figure 1a), the Rapid Atrial Pacing group exhibited partial cellular degeneration, arranging in disorder, and interstitial proliferation (Figure 1b); and the myocardial cells arranged more compact in the group treated with resveratrol and apocynin (Figure 1c,d).

Resveratrol inhibited the protein of the calpain and gp91phox, but improved cTnT.

The expression of calpain was increased 1.114 ± 0.038 fold in the rapid pacing group compared to the control group (P<0.05), however, compared to the rapid pacing group, it decreased 0.888 ± 0.022 fold in the apocynin group and 0.927 ± 0.026 fold in the resveratrol group (P<0.05). The expression of gp91phox was increased 0.452 ±

Groups	MDA(mmol/mgprot)	SOD(U/mgprot)	ROS(μmol/m)
Control	2.581± 0.344	232.000±3.010	0.004±0.001
Rapid pacing atrial	5.812 ± 0.107*	202.202±4.002*	0.021±0.006*
Apocynin	3.707±0.385*#	224.001±1.000*#	0.010±0.003*#
Resveratrol	3.548±0.437*#	221.800±2.501*#	0.012±0.002*#

*P < 0.05, compared with control group; # P < 0.05, compared with rapid pacing group

Table 1: The levels of SOD, MDA and ROS in different groups (means ± SD, n = 6)

Groups	NADPH oxidase(×10³/μmol NADPH/min/mg)
Control	2.040±0.421
Rapid pacing atrial	63.422±5.180*
Apocynin	12.450±0.882*#
Resveratrol	17.460±1.801*#

*P<0.05, compared with control group; # P<0.05, compared with rapid pacing group

Table 2: The activities of NADPH oxidase in different groups (means ± SD, n = 6)

0.008 fold in the rapid pacing group compared to the control group (P<0.01), however, compared to the rapid pacing group, it decreased 0.336 ± 0.009 fold in the apocynin group and 0.348 ± 0.001 fold in the resveratrol group (P<0.01). The expression of cTnT decreased 0.168 ± 0.005 fold in the rapid pacing group compared to the control group (P<0.01), (Figure 2) however, compared to the rapid pacing group, it was increased 0.540 ± 0.023 fold in the apocynin group and 0.506 ± 0.020 fold in the resveratrol group (P<0.05).

Resveratrol inhibited the mRNA of the calpain and gp91phox, but improved cTnT.

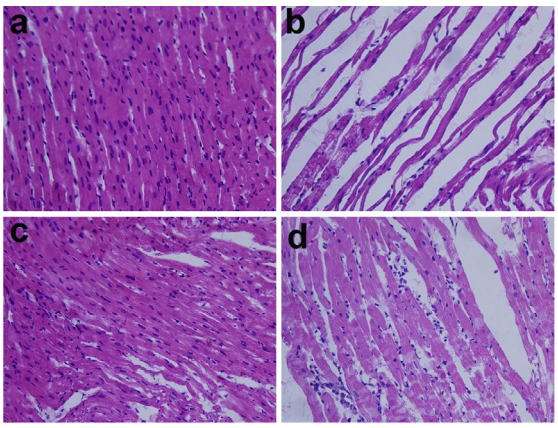
The mRNA expression was significantly different in the four groups (Figure 3). In the resveratrol group, a low basal level of calpain, p22phox and gp91phox mRNA expression was detected; in the rapid pacing group, a high level of the calpain, p22phox and gp91phox mRNA expression was quantified.

Compared with the Ctrl group, the expression of calpain, gp91phox and p22phox were higher, but the cTnT was lower (P<0.05) in the RAP group; With the RAP group, the expression of calpain, gp91phox and p22phox were lower, but the expression of the cTnT was lower (P<0.05) in the RES group; With the Ctrl group, the index of oxidative stress injury were higher, the expression of SOD was lower(P<0.05) in the RAP group; With the RAP group, the index of oxidative stress injury were lower, the expression of SOD was higher(P<0.05) in the RES group.

Discussion

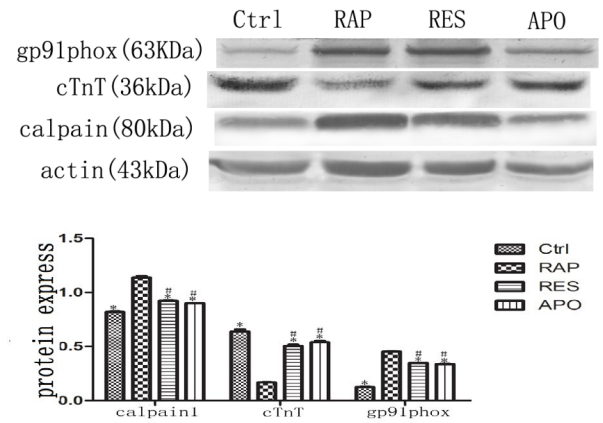
The main findings of this study were as follows: 1) NADPH-dependent superoxide production from the right atrial homogenates is increased in the rapid pacing atrial group in the absence of changes in the protein and the mRNA expression of the p22phox and gp91phox nox2 subunits; 2) Calpain activation is mediated by NADPH oxidase-dependent mechanisms in the rapid pacing atrial cardiomyocytes; 3) Resveratrol may be effective against atrial fibrillation, by virture of its potent inhibitory effect of a major oxidative system (i. e. NADPH oxidase).

During the rapid pacing atrial, the serum markers of oxidative stress from the rabbit's atrial tissue samples were higher than the control group; the expression of main NADPH oxidase subunits and calpain were more increased at the protein level and the messenger level. Among these sources, NADPH oxidase are considered to be unique because they generate ROS in a highly regulated manner and



a: Control Group; b: Rapid Pacing Group; c: Apocynin Group; d: Resveratrol Group

Figure 1: Results of histopathology study (*400)

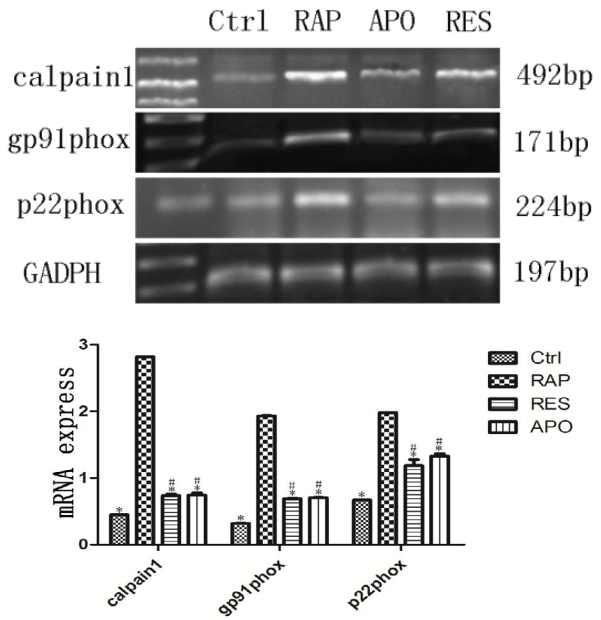


a: Control Group, b: Rapid Pacing Group, c: Apocynin Group, d: Resveratrol Group *P < 0.05, compared with control group, # P < 0.05, compared with Rapid Pacing Group

Figure 2: Results of western blotting study

can amplify oxidative stress [18-23]. Our findings suggest that ROS derived from NADPH oxidase, especially Nox2 and Nox4 subunits, contributes to tachypacing-induced myofibril degradation [24-26]. Increased superoxide production can damage various components responsible for cellular energetic and ionic homeostasis, such as phosphor transferase enzymes and ion channels, eventually interfering with myocyte structure and function [27] and, thus, can have deleterious effects on myocardial cells [28,29]. It is possible that there are common signaling cascades in mediating oxidative stress-induced pathological changes either at the atrial level.

A recent study demonstrates that numerous factors contribute to atrial hypocontractility, including action potential shortening, I_{CaL}-reduction, disturbed Ca^{2+} wave propagation, abnormal Ca^{2+} handling, and altered myofibril function [30]. Several clinical and experimental studies indicate that myofibril loss or degradation is an important factor in promoting AF-related atrial hypocontractility [31-35]. Calpain-activity-on is also known to contribute to tachycardia-induced myofibril degradation and atrial hypo contractility. In this study, we find that inhibition of oxidative stress and attenuate tachypacing-stimulated calpain activation and myofibril degradation. This finding



a: Control Group, b: Rapid Pacing Group, c: Apocynin Group, d: Resveratrol Group *P < 0.05, compared with Control Group: # P < 0.05, compared with Rapid Pacing Group

Figure 3: Results of RT-PCR study

agrees with the study at the ventricular level showing that and oxidative stress contribute to ventricular contractile dysfunction via alterations in Ca^{2+} handling proteins [36-39]. Oxidative stress may become interesting therapeutic targets to prevent AF-induced structural re modeling.

The small polyphenol resveratrol, found in various plant-derived sources, including grapes, is considered to contribute to the beneficial effect of red wine in cardiovascular diseases [40-42]. The results of the present study indicate that resveratrol inhibit NADPH oxidase activity but did not inhibit their production.

Conclusion

This study shows that resveratrol attenuates AF promotion by atrial tachycardia in isolated rabbit hearts, supporting a strong association between resveratrol and NADPH oxidase activity.

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