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Variance of DDAH/PRMT/ADMA pathway in atrial fibrillation dogs

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ABSTRACT

Atrial fibrillation (AF) may cause thrombus formation in the left atrial appendage (LAA). Thrombus formation is associated with LAA endocardial dysfunction. Because asymmetrical dimethylarginine (ADMA) can cause endothelial dysfunction by decreasing nitric oxide (NO) formation, we investigated plasma ADMA and nitrite/nitrate (NO_X) levels and myocardial dimethylarginine dimethylaminohydrolase-2 (DDAH-2), protein arginine methyltransferase-1 (PRMT-1), and endothelial NO synthase (eNOS) protein contents from AF dogs. The results displayed that plasma ADMA level significantly increased, and plasma NO_X concentration significantly decreased. Compared with normal heart, DDAH-2 expression was unchanged in the fibrillating atria. However, the DDAH activity was significantly decreased in the fibrillating atria. PRMT-1 expression significantly increased in the LAA and in the left atrium (LA). ENOS expression significantly decreased in the LA. ENOS and PRMT-1 expressions were unchanged in the right atria. Our results suggested that the DDAH–PRMT–ADMA system maybe play a pivotal role in regulating endothelial function in AF.

Atrial fibrillation (AF) is the most common cardiac arrhythmia encountered in clinical practice. AF is associated with an increased stroke incidence which is mainly caused by thrombus formation in the left atrial appendage (LAA). The mechanisms responsible for localized LAA thrombus formation remain unclear. Recently, Cai et al. have shown that AF caused a decrease in nitric oxide (NO) production in the left atrial (LA) and LAA endocardium [1]. NO in the endocardium is synthesized by the endothelial nitric oxide synthase (eNOS) and has powerful anti-thrombotic properties. However, no changes in eNOS protein expression in the LAA have been reported in AF [1].

Accumulating data indicated that NO production may be regulated by asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor [2–5]. Therefore, it is possible that the reduction in endocardial NO formation in AF is due to the increased inhibition of eNOS by ADMA. ADMA is synthesized endogenously during the methylation of protein arginine residues by protein arginine methyltransferases (PRMTs) [4]. Two types of PRMT, namely PRMT type I and PRMT type II, have been found. PRMT type I forms ADMA and *N*-monomethylarginine (NMA), whereas PRMT type II forms symmetric dimethylarginine (SDMA) and NMA [5]. PRMT-1 is the predominant isoform of PRMT type I in mammalian cells [6]. In addition, ADMA can be degraded by the dimethylarginine dimethylaminohydrolases (DDAH-1 and DDAH-2) [4,7]. However, DDAH-1 and DDAH-2 exist with distinct tissue distribution. DDAH-1 predominates in tissues that express neuronal NOS. DDAH-2 predominates in tissues expressing endothelial NOS. In brief, ADMA accumulation may be associated with abnormal expression and/or activity of ADMA produced and/or degraded enzyme. So, we hypothesized that the LAA endocardial dysfunction in AF could be related to the decreased DDAH-2 protein expression and/or depressed DDAH activity and increased PRMT-1 protein expression. This hypothesis is supported by reports that plasma ADMA level increased in the setting of AF [8–10]. Consequently, this study was carried out to determine DDAH activity, DDAH-2 and PRMT-1 protein contents in the normal and fibrillating animal heart.

Materials and methods

Experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at Harbin Medical University.

AF animal model. Mongrel dogs of either sex (weighing 17–21 kg; AF group; $n = 7$) were obtained from the Animal Center of Harbin Medical University. The operation was performed using sterile surgical techniques. The dogs were anaesthetized with sodium pentobarbital (30 mg/kg, iv). A right fifth intercostal thoracotomy was performed, and the pericardium was opened. A spiral epicardial electrode (Medtronic Inc., USA) was sutured to

Abbreviations: DDAH, dimethylarginine dimethylaminohydrolase; PRMT, protein arginine methyltransferase; ADMA, asymmetrical dimethylarginine

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the tip of right atrial appendage and connected to a pacemaker (Electronic Engineering, Department of Fudan University Instruments, China) in subcutaneous pockets in the right chest. The chest wall was closed in layers. The dogs were allowed 7 days to recover before pacing at a rate of 400 bpm for 6 weeks. Digoxin (0.25 mg per day) was given to control ventricular rate [11]. Surface electrocardiograms (ECG) were performed periodically to ensure atrial pacing and well-controlled ventricular rate.

To compare protein expression, six-shamed operated and nonpaced mongrel dogs were used as controls (control group). After 6-week pacing, all animals including control group were once again anesthetized and the hearts were rapidly excised through a right lateral thoracotomy. Samples from the right and left atria and atrial appendages were obtained. These were placed and frozen in liquid nitrogen for subsequent Western analysis and immunohistochemical staining.

Detection of plasma ADMA and nitrite/nitrate concentration. Plasma concentration of ADMA was determined by high-performance liquid chromatography (HPLC) as previously described [12,13] with the following modification. In brief, blood samples were collected in EDTA tubes. The plasma was separated at 4 °C by centrifugation at 2000 rpm for 10 min. The plasma (200 μL in each example) was disposed with acetonitrile (400 μL in each example) for 5 min. The supernatant was separated at 4 °C by centrifugation at 12,000 rpm for 10 min. *O*-Phthalaldehyde mercaptoethanol derivatives of the supernatant were used for detection of ADMA. A gradient mobile phase, consisting of sodium phosphate buffer (pH 3.5) and methanol (58:42, V/V), was used at a flow rate of 1.0 mL/min at room temperature. Plasma ADMA was analyzed using detection fluorescence with excitation wavelength (λ_{ex}) at 338 nm, emission wavelength (λ_{em}) at 425 nm.

Plasma nitrite/nitrate (NO_X) was assayed with a NO_X Detection Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Briefly, blood samples were collected in the tubes containing heparin. The plasma was separated at 4 °C by centrifugation at 2000 rpm for 5 min. Nitrate reductase enzyme was used to convert nitrate to nitrite. Plasma NO_X level was estimated using the Griess reagent and absorbance was read at 540 nm.

Measurement of eNOS, PRMT-1, and DDAH-2 protein levels. ENOS, PRMT-1, and DDAH-2 protein expressions were measured by Western blotting as previously described [1,14]. Briefly, freshly isolated cardiac tissue samples were weighed and homogenized on ice in homogenization buffer (50 mmol/L Tris, 150 mmol/L NaCl, 5 g/L a-Cholate sodium, 1 g/L SDS, 2 mmol/L EDTA, 10 g/L Triton X-100, 100 mL/L glycerin, 1 mmol/L PMSF, and 2 mg/L aprotinin). The protein concentration in the supernatant was determined using the Lowry Assay. Equal amounts of protein samples (45 μg) were separated in 8% SDS–PAGE for eNOS assay and 12% SDS–PAGE for PRMT-1 and DDAH-2 assay, and transferred onto nitrocellulose membrane at 220 mA for 2 h. Polyclonal anti-eNOS (1:1000) and anti-DDAH-2 (1:100) antibodies and monoclonal anti-PRMT-1 (1:1000) (Abcam Inc., Cambridge, UK) and anti-GAPDH (1:1000) (Santa Cruz Biotechnology, CA) antibodies were used to detect target protein expression, respectively. The integrated optical densities (IOD) of these bands were obtained by an imaging system (Image-Master VDS System, Pharmacia Biotech, Sweden). The values of eNOS/GAPDH, PRMT-1/GAPDH, and DDAH-2/GAPDH were used to express the levels of eNOS, PRMT-1, and DDAH-2 expression.

Measurement of DDAH activity. DDAH activity was assayed as previously described [15]. Briefly, the tissue homogenates were incubated with 2 mmol/L ADMA–0.1 mol/L sodium phosphate buffer (pH 6.5) in a total volume of 0.4 mL for 30 min at 37 °C. The reaction was stopped by the addition of color developing reagent [2] in a total volume of 1. 4 mL, and the supernatant was boiled for 15 min at 95 °C, and centrifuged at 11,000 rpm for 10 minutes at 4 °C. The amounts of L-citrulline formed were determined with the spectrophotometric analysis at 530 nm. As the assay blank, the enzyme preparations heated in a boiling water bath were subjected to the determinations of the activity.

Immunohistochemical staining of inducible NOS (iNOS). Immunohistochemical stain of iNOS protein was performed by using a universal two-step immunohistochemical detection kit (Beijing Zhongshan Biotechnology Co., China). In brief, 5 μM sections from paraffin embedded tissue sections were deparaffinized in xylene, and then dried and rehydrated. The endogenous peroxidase activity was eliminated by pre-treatment with 3% H_2O_2 in methanol for 5 min. The primary rabbit polyclonal antibody against iNOS at a titer of 1:100 (Abcam Inc., Cambridge, UK) was applied and incubated overnight at 4 °C. The sections were washed in PBS and then incubated with a secondary goat anti-rabbit antibody-HRP (Power Rision Two-step Histostaining Reagent) for 25 min. After that the reaction was developed by using DAB. Finally, slides were counterstained with hematoxylin, and analyzed by using image software.

Statistical analysis. Data are presented as means ± SEM. Comparisons between AF and control groups were performed using Student's *t* test for unpaired data. Pearson's correlation coefficients (r) and associated P values were calculated. $P < 0.05$ was considered statistically significant.

Results

ECG confirmed an atrial paced rhythm and the presence of AF in each animal of the AF group and sinus rhythm in each animal of the control group. There was no difference in ventricular rate between AF and control groups (ventricular rate 148 ± 13 bpm versus 142 ± 17 bpm, P = NS).

ADMA and NO_X assay

The concentrations of plasma ADMA and NO_X were determined in between AF and control groups at the end of the experiment. Compared with control group, plasma ADMA significantly increased (2.15 ± 0.12 versus 1.05 ± 0.06 μmol/L; $P < 0.05$), and plasma NO_X significantly decreased in AF group (30.6 ± 3.4 versus 51.9 ± 7.8 μmol/L; $P < 0.05$).

To determine the relationship between ADMA and NO_X in AF, we plotted the individual values of ADMA and NO_X. As expected, an inverse correlation existed between plasma ADMA and NO_X levels, NO_X concentration being related to ADMA level by the following formula: NO_X concentration = −9.69 ADMA + 59.79 (r = −0.55; $P < 0.05$).

Down-regulation of eNOS expression in AF

ENOS expressions from both AF and control groups were quantified by Western analysis by using a rabbit polyclonal antibody against eNOS (Fig. 1A). ENOS protein expression in the LA of the AF group was 53% less than that in control group (0.34 ± 0.05 versus 0.72 ± 0.08, $P < 0.05$, Fig. 1B). But, there was no significant difference in eNOS expression in the right atrium (RA) between AF and control groups (0.65 ± 0.07 versus 0.78 ± 0.09, P = NS, Fig. 1B), as well as in the LAA and right atrial appendage (RAA).

Up-regulation of PRMT-1 expression in AF

The level of PRMT-1 expression was determined by Western analysis by using a mouse monoclonal antibody (Fig. 1A). The expression of PRMT-1 increased by approximately 56% in the LA and by approximately 35% in the LAA in the AF group compared