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心房顫動引發心房組織 Annexin II 之定量及其心房心肌細胞內分佈變化-心房低纖維蛋白溶解可能機轉
研究成果報告(精簡版)

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Down-Regulation of Cardiac Annexin II Levels in Fibrillating Human Atria without Significant Mitral Valve Disease: Potential Mechanisms for Atrial Hypofibrinolysis

Running title: Annexin II in Atrial Fibrillation

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Background: Patients with atrial fibrillation (AF) in addition to their hypercoagulable state have also been found to have impaired fibrinolytic function. Recently, annexin II, a member of calcium-dependent phospholipids binding proteins, has been identified on endothelial cells and found to serve as a platform on the surface of the endothelial cell for the binding of plasminogen and tissue plasminogen activator (tPA), as well as to enhance plasmin generation thereby promoting fibrinolysis.

Methods: To understand whether the regional hypofibrinolysis was correlated with annexin II expression and whether the latter was related to endothelial dysfunction, we studied the annexin II protein and gene expression in left atrial appendage, markers of left atrial fibrinolytic activity and markers of endothelial dysfunction in 20 patients with chronic non-rheumatic AF > 6 months and in 20 matched controls without AF (i.e., sinus rhythm, SR) undergoing coronary artery bypass surgery. Left atrial and peripheral venous levels of tissue plasminogen activator (tPA) antigen, von Willebrand's factor, plasmin-antiplasmin complex (PAP), and plasminogen activator inhibitor type I (PAI-I) activity were measured by enzyme linked immunosorbent assay. The annexin II protein levels were measured by western blotting.

Results: Western blot analyses demonstrated a significant decrease (90%) in annexin II protein levels in the AF group as compared to SR patients (0.04±0.01 vs 0.44±0.06; $p < 0.01$). In AF group, we found a significant positive correlation between annexin II and PAI activity (r=0.51, P<0.01). There were significant negative correlations between annexin II and von Willebrand's factor (r=-0.64, P<0.01) and between annexin II and tPA antigen (r=-0.42, P<0.05). Significant positive correlations between PAP

complex and annexin II (r=0.75, P<0.01) were found.

Conclusions: We conclude that chronic non-valvular AF is associated with a down-regulation of annexin II, which may contribute to the pathophysiology of a hypofibrinolytic state and endothelial dysfunction in the diseased atria.

Key Words: atrium; annexin; fibrillation; fibrinolysis

Introduction

Atrial fibrillation (AF) is the most common underlying cardiac disorder predisposing to systemic embolism (1). Patients with chronic AF are at risk of stroke with thromboembolism usually originates in the left atrial cavity, and more than 90% of left atrial thrombi are located in the appendage (2). Although decreased flow and hypercoagulable state of the blood in the fibrillating atrium have been emphasized as the underlying mechanisms, endocardial dysfunction and impaired fibrinolytic function/activity in maintaining the local coagulation and fibrinolytic balance could also contribute to the thrombogenesis in AF (3).

Annexin II belongs to a family of calcium-dependent, phospholipid-binding proteins from which at least 10 different species have been described (4, 5). Among the 13 isoforms, annexin II exists in the mammalian myocardium in the high amounts and the existing experimental evidence suggests that annexin II in its tetrameric form is the main physiological receptor for the plasmin precursor plasminogen and plasminogen tissue plasminogen activator on the extracellular surface of endothelial cells (6). Considering the hypercoagulable state in fibrillating atria, the possibility of modulation of fibrinolytic activity by annexin II might be of great interest. We hypothesize that down-regulation of endothelial membrane annexin II impairs the appropriate formation of the plasminogen/tissue plasminogen activator/annexin II complex, disrupting a key regulatory mechanism in fibrinolytic vigilance. This would in turn produce decreased fibrinolytic activity and indirectly promote a thrombophilic state in diseased atria.

Therefore, the purpose of the present study was (1) to investigate whether the gene and protein expression of atrial annexin II altered in chronic AF, (2) to determine whether regional levels of plasma markers of fibrinolytic activity and endothelial dysfunction changed during AF, and (3) to determine the correlations between the regional hematologic indexes and the expression of annexin II protein in LAA in AF cohorts.

METHODS

Patients

Light atrial appendages (LAA) were obtained from 40 patients who underwent first-time coronary-artery bypass grafting (CABG). Patients with good ventricular function requiring triple vessel bypass grafting (left anterior descending coronary artery plus two other coronary arteries) were recruited. Tissue samples (1 or 2 entire cross sections, depending on the amount of tissue available) were taken from 20 patients with non-rheumatic persistent AF (duration > 6 months, confirmed electrographically) and from 20 matched patients with no history of AF. Patients included in the study had to be older than 18 years of age and scheduled for elective CABG requiring cardiopulmonary bypass. All of the patients were in stable cardiac condition before surgery. The study was approved by the local research ethics committee and conducted in accordance with the Declaration of Helsinki and with the written informed consent of all volunteers. Patients were excluded if they had paroxysmal AF, or if they had acute coronary syndrome, recent venous thrombosis or a history of systemic embolism, uncontrolled renal, hepatic, or heart failure.

Clinical and hemodynamic parameters

The clinical history was obtained by history taking before surgery. Presence, type, and duration of AF were assessed according to the previous serial electrocardiograms. For all patients, an evaluation of left ventricular function by echocardiography or left ventricular angiogram had been obtained. The right atrial pressure and pulmonary wedge pressure were obtained from preoperative cardiac catheterizations and presented the right and left atrial filling pressure, respectively.

Blood sampling and measurements

Taking aspirin $\leqq$ 100 mg/day was not a contraindication. Warfarin and other anticoagulation drugs were discontinued at least 5 days before CABG. Blood collection was performed simultaneously from the left atrium and peripheral vein at the time of atrial cannulation before cardiopulmonary bypass according to acknowledged procedures and rigorously standardized to avoid diurnal variations in fibrinolytic activity. Blood samples were drawn atraumatically and without stasis into syringes preloaded with trisodium citrate (0.011 mol/L final concentration). After collection of blood samples, platelet-poor plasma fractions were obtained by centrifugation at 4° C for 20 minutes at 2200 g (within 5 minutes after blood collection) and stored at –20° C for further investigations. Plasma von Willebrand's factor (vWF) concentrations were measured using immunoturbidimetric assay (Diagnostica Stago, France). Markers of the fibrinolytic system were tissue-type plasminogen activator (tPA) antigen and its inhibitor (PAI-1) activity, measured with an enzyme-linked immunosorbent assay (ELISA) techniques (Zymutest tPA antigen and PAI-1 activity ELISA kit, Hyphen Biomed,

Neuville-Sur-Oise, France). Fibrinolytic activation was assayed by measuring plasmin-antiplasmin complexes (PAP complex ELISA kit, Technoclone , Vienna, Austria). Intra- and interassay coefficients of variation for all assays were less than 5% and 10%, respectively.

Western blot analysis

The relative protein levels of annexins II in cardiac homogenates from LAA were assessed using the Western blot technique. In brief, 40 µg of atrial tissue extracts were subjected to electrophoresis under denaturing conditions using a sodium dodecyl sulphate (SDS) buffer system in 10% SDS polyacrylamide gels (SDS-PAGE). After electrophoretic separation, proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham) using a Hoefer Scientific Instruments Transphor Units at 150 mA for 2 h. Membranes were then successively incubated in blocking buffer containing 100 mM Tris-HCL (pH 7.5), 0.9% (w/v) NaCL, 0.1% (v/v) fetal bovine serum for 2 h at room temperature. Antibodies against annexin II (1:500) and α-tubulin (1:500) were diluted to the corresponding concentrations in an antibody binding buffer containing 100 mM Tris-HCl (pH 7.5), 0.9% (w/v) NaCL, 0.1% (v/v) Tween-20, and 1% (v/v) fetal bovine serum. These mixtures were then incubated for 3.5 h at room temperature. After incubation with primary antibody, immunoblots were washed in triplicate in 50 ml blotting buffer for 10 min and then immersed in the secondary antibodies against rabbit IgG (Promega; 1:1000) for 1 hour. After incubation with primary antibody, membranes were washed three times for 5 min in TBS and incubated fr 1 h at 4°C with a horseradish peroxidase labelled secondary antibody (BD Biosciences: 1:2000). Peroxidase activity was detected on X-ray film

using the ECL dectection reagents (Amersham). Color development was presented in a 20-ml mixture consisting of 3% H_2O_2 nitro blue tetrazolium, 10 mg 3'-3'-diaminobenzidine, 100mM NaCl, and 5 mM $MgCl_2$ in 100 mM Tris-HCl (pH 9.5). Each time different exposures were scanned and analysed using a LAS-1000 plus scanning system (Fujifilm, Japan) in combination with IR-LAS-1000 software package (Lite version). The resulting images were densitometrically analysed. The β-actin data were used as an internal standard to normalize annexin II data. The mean relative absorption units of the sinus rhythm group were compared with the corresponding means of the AF groups. Comparison of the different groups was only done only on blots processed equally and exposed on the same x-ray film. Densitometric quantification for comparison of the different groups was performed only on blots processed equally and exposed on the same x-ray film.

Statistical Analysis

All SDS-PAGE procedures were performed in duplicate series. Data are presented as mean ± SD. Statistical comparison was performed using the Student's two tailed *t* test for unpaired data for normal distributions, Mann–Whitney two sample rank test for nonparametric distributions and χ^2 analysis for group comparison. Correlation between variables was evaluated using Spearman's rank correlation method. Tests of significance were two-sided, and $P < 0.05$ was assumed as significant.

Results

Clinical characteristics and hemodynamic data

The clinical characteristics are shown in Table 1. There were 23 men and 17 women with a mean age of 67 ± 10 years (range 48 to 80). Patients with AF did not differ from control subjects with sinus rhythm with respect to clinical parameters that were evaluated except for the diameter of left atrium, which was larger in the patients with AF. Besides, there were no differences with regard to age, gender, prothrombin time and active partial thromboplatin time, clinical diagnosis of accompanied disease, and preoperative hemodynamic parameters between groups.

Annexin II protein expression

Changes in protein expression were determined by Western blotting for annexin II in relation to protein level of ß-actin. To verify the existence of annexin II at the protein level, western blot analysis of atrial tissue lysates was performed using mouse monoclonal antibodies. The specific bands labeled with anti-annexin antibodies correspond to 36 kDa for annexin II. In contrast to fibrillating atria, annexin II proteins were expressed abundantly in LAA tissue in patients with SR. In AF subjects, annexin II protein level was 90% lower than that in the SR atrial tissues (0.04±0.01 vs 0.44±0.06, $p < 0.01$). However, no differences in ß-actin densities were found between groups.

Peripheral venous versus left atrial fibrinolytic variables

Compared with SR group (tPA antigen, 10.7 ± 5.8 ng/ml; PAI-1 activity, 7.7 ± 3.6 ng/ml), tPA antigen level and PAI-1 activity was significantly higher in CAF (tPA, 23.7 ± 15.6 ng/ml; PAI-1 activity, 23.7 ± 13.4 ng/ml; both $p < 0.05$). In contrast, patients in the CAF group had a lower PAP complex compared to the SR group. In addition, the tPA, PAP complex concentrations, and PAI-1 activity in the

left atrial and peripheral venous samples of the SR group were identical (all $P < 0.05$). The tPA antigen, PAP complex concentrations, and PAI-1 activity in the left atrium and peripheral venous samples of the AF group were statistically different from those in the peripheral venous blood ($P < 0.05$).

Correlation between annexin II and fibrinolytic variables

In AF group, we found a significant positive correlation between annexin II and PAI activity (r=0.51, P<0.01). There were significant negative correlations between annexin II and vWF (r=-0.64, P<0.01) and between annexin II and tPA antigen (r=-0.42, P<0.05). Finally, significant positive correlations between PAP complex and annnexin II (r=0.75, P<0.01) were found.

Discussion

Main findings

The major findings of this study were as follows: (1) There were increased local levels of tPA antigen, PAI-1 activity and decreased levels of PAP complex in fibrillating atria. (2) Chronic AF was associated with down-regulation of annexin II in LAA. (3) The annexin II expression of LAA was negatively correlated with the markers of hypofibrinolysis and endothelial dysfunction in AF cohort.

The possible role of annexin II in fibrinolytic function during AF

The regulated function of the fibrinolytic system is fundamental to the solubilization of fibrin-containing thrombi and to a number of other biologic processes. Cell surface receptors may support fibrinolytic surveillance in both intravascular and extravascular locations by stimulating

plasmin generation and by protecting plasmin from its inhibitors. One such receptor is annexin II, a calcium- and phospholipid-binding protein that serves as a profibrinolytic co-receptor for tissue plasminogen activator and plasminogen on endothelial cells (7-9). Annexin II was first identified as an intracellular protein and attributed intracellular functions (10, 11). Even though it lacks a signal peptide and its mechanism of secretion is unknown, extracellular annexin II has recently been found in several tissues as both soluble and membrane-bound protein (12, 13).

Annexin II has been shown to accelerate the activation of the clot-dissolving protease plasmin by complexing with the plasmin precursor plasminogen and with tissue plasminogen activator. Formation of the plasminogen/tissue plasminogen activator/annexin II complex may be a vital regulatory mechanism in fibrinolytic vigilance. The existing experimental evidence suggests that annexin II in its tetrameric form is the main physiological receptor for plasminogen on the extracellular surface of endothelial cells (14, 15). This finding, together with the negative relationship between the annexin II expression and markers of the regional fibrinolytic activity/function and endothelial dysfunction in the present study, indicate that annexin II may play a central roles in the hypofibrinolytic state of fibrillating atria. If this critical mechanism is altered by AF, as we suggest here, it is plausible to postulate that the delicate balance of coagulation/fibrinolysis would be tilted towards enhanced coagulation and thrombosis.

Conclusions

For the first time, we report that chronic non-valvular AF is associated with a down-regulation of annexin II, which may contribute to the pathophysiology of a hypofibrinolytic state and endothelial dysfunction in the long-lasting diseased atria.

Acknowledgments

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