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Original Article

Positive relationship between the autoantibodies against β_1, β_2 and α_1-ARs adrenoreceptors and the development of chronic cardiorenal syndrome

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Abstract: Purpose: Previously, we demonstrate that the presence of autoantibodies against β_1, β_2 and α_1 adrenoreceptors are highly prevalent in heart failure and may participate in its pathogenesis. The purpose of the current study is to determine the relationship between the presence of autoantibodies against β_1, β_2 and α_1 adrenoreceptors and chronic cardiorenal syndrome (CRS). Methods: Synthetic peptides corresponding to amino acid sequences of the second extracellular loops of β_1, β_2 and α_1 adrenoreceptors were synthesized as antigens to test 30 patients with chronic CRS, 30 heart failure patients without kidney disease and 38 healthy controls for the presence of autoantibodies using enzyme-linked immunosorbent assay. Results: The respective frequencies of autoantibodies against β_1, β_2 and α_1 adrenoreceptors are 66.6% (20/30), 73.3% (22/30) and 70% (21/30) in patients with chronic CRS, 40.0% (12/30) (P=0.04), 46.7% (14/30) (P=0.03) and 43.3% (13/30) (P=0.03) in heart failure patients without kidney disease and 10.5% (4/38), 7.9% (3/38) and 10.5% (4/38) (P < 0.001) in healthy controls. Titers of these autoantibodies are also significantly increased in patients with chronic CRS. Meanwhile the titers of three autoantibodies have positive correlation with the increase of the value of serum creatinine. Conclusions: This study demonstrates that the presence and titers of autoantibodies against β_1, β_2 and α_1 adrenoreceptors in patients with chronic CRS are not only significantly increased than those of healthy control group, but also significantly increased than those of heart failure group. We posit that these autoantibodies may be involved in the pathogenesis of chronic CRS.

Keywords: Cardiorenal syndrome, adrenoreceptor, autoantibody

Introduction

Cardiac and renal diseases are common and frequently coexist to significantly increase mortality, morbidity and the complexity and cost of care. Cardio renal syndrome (CRS) is a disorder affecting both the heart and kidneys whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction in the other. We identified five sub-types of the syndrome [1, 2]: Acute cardio-renal syndrome (type 1), Chronic cardio-renal syndrome (type 2), Acute reno-cardiac syndrome (type 3), Chronic reno-cardiac syndrome (type 4), and secondary cardio-renal syndrome (type 5).

Chronic cardio-renal syndrome (type 2) means chronic abnormalities in the heart function leads to kidney injury or dysfunction. This subtype refers to a more chronic stage of kidney disease resulting in the complication of chronic heart disease. This syndrome is common and has been reported in 63% of patients hospitalized with congestive heart failure (CHF) [1, 3, 4]. Currently, the factors and mechanisms involved in the pathogenesis of CRS (type 2) remain poorly understood.

Previously, we demonstrated that the presence of autoantibodies against β_1, β_2 and α_1 adrenoreceptors (anti-β_1, β_2 and α_1-ARs), which is

Table 1. Amino acid sequences of human β_1, β_2 and α_1-adrenoreceptors

Adrenoreceptor	Position	Sequence
β_1	197-222	H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R
β_2	172-197	H-W-Y-R-A-T-H-Q-E-A-I-N-C-Y-A-N-E-T-C-C-D-F-F-T-N-Q
α_1	192-218	G-W-K-E-P-V-P-P-D-E-R-F-C-G-I-T-E-E-A-G-Y-A-V-F-S-S-V

The purity of the synthesized peptides corresponding to the amino acid sequence of the second extracellular loop of human β_1, β_2 and α_1 adrenoreceptors, determined by high performance liquid chromatography (HPLC) using a Vydac C-18 column, was 96.66%, 96.21% and 95.34%. The molecular weight of peptides corresponding to the amino acid sequence of the second extracellular loop of human β_1, β_2 and α_1 adrenoreceptors was analyzed by mass spectrometry and the molecular weight was 3484.9, 3237.5 and 2872.2.

bounded to the second extracellular loop of the receptors, highly prevalent in heart failure and may participate in its pathogenesis [5, 6]. The first aim of this study is to investigate differences in the frequencies of anti-β_1, β_2 and α_1-ARs among patients with chronic CRS, compared to heart failure patients without kidney disease and healthy controls. The second aim is to investigate the relationship between the presence of anti-β_1, β_2 and α_1-ARs and the value of serum creatinine of chronic CRS.

Patients and methods

Experimental design and subjects

This trial was carried out at Capital Medical University Beijing Chao-Yang Hospital. The research protocol was approved by the Medical Ethics Committee of our hospital. All the patients provided were informed and consent obtained before recruitment. 30 patients were diagnosed with chronic CRS based on the criteria set by the National Kidney Foundation Report [3], the American College of Cardiology Foundation/American Heart Association Report [7], and the Claudio Ronco, et al Report [1]. The criteria include glomerular filtration rate (GFR) ≤ 60 mL/min per 1.73 m^2 with previously left ventricular ejection fraction (LVEF) ≤ 45%. We selected two comparison groups: 30 heart failure patients without kidney disease (heart failure group, HF group) and 38 healthy persons (healthy control group). Exclusion criteria for all the groups were diabetes mellitus, vasculitis and autoimmune disease. Each patient's data were recorded in accordance with the protocol. Blood samples were collected from antecubital veins at recruitment by tubes containing EDTA, and centrifuged at 2000 rpm for 10 minutes at 4°C within 2 h of the collection. Serum samples were stored at -70°C until the assay was performed.

Materials

Three kinds of peptides corresponding to the amino acid sequence of the second extracellular loop of human β_1, β_2 and α_1 ARs were synthesized by Genomed (Genomed Synthesis, Inc., CA, and USA) and the sequences were shown in **Table 1** [8-10]. The purity of the synthesized peptides corresponding to the amino acid sequence of the second extracellular loop of human β_1, β_2 and α_1 ARs, determined by high performance liquid chromatography (HPLC) using a Vydac C-18 column, were 96.66%, 96.21% and 95.34%. The molecular weight of peptides corresponding to the amino acid sequence of the second extracellular loop of human β_1, β_2 and α_1 ARs were analyzed by mass spectrometry and the molecular weights were 3484.9, 3237.5 and 2872.2. Nunc microtiter plates were purchased from Kastrup, Denmark. Tween-20, thimerosal, and ABTS were purchased from Sigma, St. Louis, MO, USA. Fetal bovine serum, biotinylated goat anti-human IgG (H+L), and horseradish peroxidase-streptavidin were bought from Zhongshan Golden Bridge Biotech, Beijing, China. The microplate reader was purchased from Molecular Devices Corp, Menlo Park, CA.

ELISA protocol

Samples were classified into positive or negative based upon the presence or absence of anti-β_1-AR, anti-β_2-AR, and anti-α_1-AR. An ELISA protocol, previously described by Fu *et al* [11], was used to screen for the presence of the autoantibodies. Briefly, 50 mL of peptide (5 mg/L) in 100 mmol Na_2CO_3 solution (pH=11) was coated on microtiter plates overnight at 4°C. The wells were saturated with PMT (1× PBS, 1 mL/L Tween-20, and 0.1 g/L thimerosal (PBS-T) supplemented with 100 mL/L fetal bovine serum) for 1 hour at 37°C. Then positive control and negative control with 50 mL of serum diluted from 1:20 to 1:160, was added to the wells for 1 hour at 37°C. After washing the wells with PBS-T three times, affinity-puri-

Table 2. Clinical characteristic of subjects from three groups in the present study

	Healthy control (n=38)	Heart failure (n=30)	Chronic cadiorenal syndrome (n=30)
Age (years)	60.48±9.10	62.48±10.05	68.61±10.14
Weight (Kg)	68.63±10.10	70.54±10.20	73.63±12.00
NT-proBNP (pg/mL)	563.79±129.23	9076.48±1035.56***	15696.96±13446.33###
LVEF (%)	68.45±3.01	38.00±4.15***	36.30±5.23&&&
Scr (μmol/L)	78.67±19.43	92.20±23.56	277.29±237.82&&&,###
Ccr (ml/min)	89.12±7.56	83.58±3.48	33.01±16.47&&&,###
GFR (ml/min/1.73 m^2)	101.72±10.10	94.35±9.12	28.64±15.05&&&,###

Mean ± SD are shown. Student's unpaired two-tailed T-test was made between heart failure patients vs. healthy control, cardiorenal syndrome patients vs. heart failure patients and cardiorenal syndrome patients vs. healthy control, and there are significant differences. ***: P < 0.001 heart failure patients vs. Healthy control; ###: P < 0.001 cardiorenal syndrome patients vs. heart failure patients; &&&: P < 0.001 cardiorenal syndrome patients vs. healthy control. LVEF: left ventricular ejection fraction; Scr: serum creatinine concentration; Ccr: creatinine clearance rate; GFR: glomerular filtration rate.

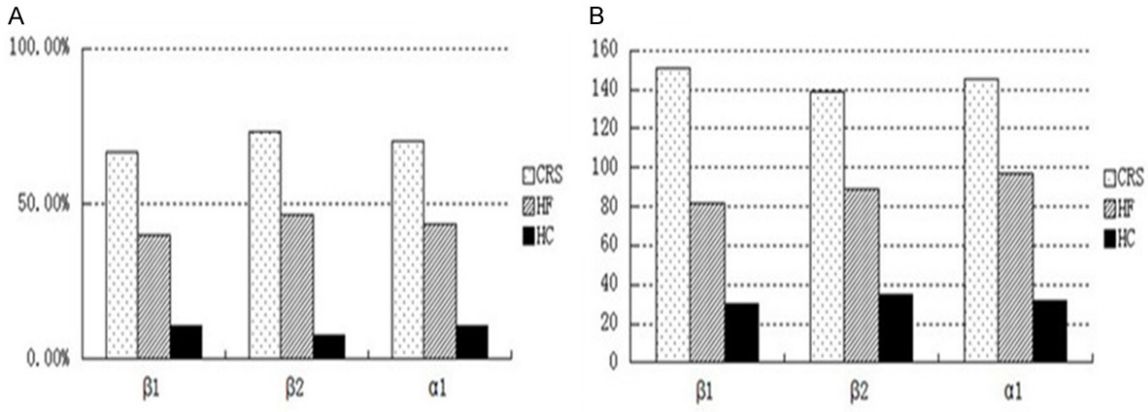

Figure 1. Frequencies (A) and geometric mean titers (B) of autoantibodies among the three groups. Frequencies and geometric mean titers of anti-β_1, β_2, and α_1-ARs were significantly higher in the chronic cardiorenal syndrome patients than in the heart failure patients and healthy controls. #: P < 0.05 cardiorenal syndrome patients vs. heart failure patients; ***: P < 0.001 cardiorenal syndrome patients vs. healthy controls; &: P < 0.05 heart failure patients vs. healthy controls. CRS: chronic cardiorenal syndrome group; HF: heart failure group; HC: Healthy control group.

fied biotinylated goat antihuman IgG (H+L) (1:500 dilution in PMT) was added for 1 hour at 37°C. Following another round of washing three times, the bound biotinylated antibody was detected by incubating the microtiter plate for 1 hour at 37°C with horseradish peroxidase streptavidin (1:500 dilution in PMT). This was followed by washing three times with PBS, the addition of 2.5 mmol/L H_2O_2, and then of 2 mmol/L ABTS in a citrate buffer solution. Absorbance (A) was measured after 30 minutes at 405 nm in a microplate reader.

Data analysis

Quantitative data was expressed as the mean ± SD. Positivity was defined as the ratio of (sample A-blank A)/(negative control A-blank A) ≥ 2.1. Antibody titer was reported as a geometric mean. Data was analyzed using SPSS 16.0. (SPSS, Chicago, Illinois, USA) Fisher's exact test and unpaired t tests were used to determine the significance in differences between groups. The correlation with autoantibodies was tested using the Spearman correlation coefficient. Association between the presence of autoantibodies and serum creatinine was assessed using multiple logistic regression analysis. P < 0.05 was considered as statistically significance.

Results

Patient characteristics

A total of 98 eligible study subjects were enrolled from March 2013 to September 2013. Patients were divided into three groups based

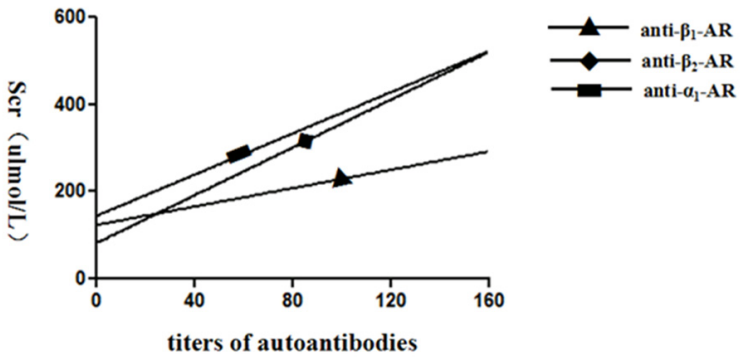

Figure 2. The relationship between titers of autoantibodies and serum creatinine. Scr: serum creatinine; anti-β_1-AR: autoantibodies against β_1 adrenoreceptor; anti-β_2-AR: autoantibodies against β_2 adrenoreceptor; anti-α_1-AR: autoantibodies against α_1 adrenoreceptor.

on their GFR and LVEF. There were 30, 30, and 38 study subjects in the chronic cardiorenal disease group, heart failure group and healthy control group respectively. There were no significant differences in the proportion of patients with hypertension or other cardiovascular risk factors. The difference between GFR and LVEF among the groups was significantly different (**Table 2**).

ELISA results

Sera positive for anti-β_1-AR was found in 66.6% (20/30) of the chronic CRS group, 40.0% (12/30) (P=0.04) of the heart failure group, and 10.5% (4/38) of the healthy control group. Sera positive for anti-β_2-AR was found in 73.3% (22/30) of the chronic CRS group, 46.6% (14/30) (P=0.03) of the heart failure group, and 7.9% (3/38) of the healthy control group. Positive sera for anti-α_1-AR was found in 70% (21/30) of the chronic CRS group, 43.3% (13/30) (P=0.03) in the heart failure group, and 10.5% (4/38) (P < 0.001) of the healthy control group. Titers of these autoantibodies were also significantly increased in patients with chronic CRS (P < 0.001), see **Figure 1A**, **1B**. With the increase of serum creatinine, titers of these autoantibodies were gradually increased (**Figure 2**).

Positive sera from the chronic CRS group contain different kinds of autoantibodies. 7 patients were positive for a single autoantibody, nine patients were positive for two kinds of autoantibody, and thirteen patients were positive for all three kinds of autoantibody. A significant correlation was found among anti-β_1-AR and anti-α_1-AR (rs=0.4, P=0.02), anti-β_1-AR and anti-β_2-AR (rs=0.6, P < 0.001). In the chronic CRS group, 55.0% (11/20) of the 20 patients with anti-β_1-AR, were also positive for anti-α_1-AR, and 70.0% (14/20) had anti-β_2-AR.

Discussion

In this study, we demonstrated for the first time that positivity for anti-β_1, β_2 and α_1-ARs is associated with chronic CRS. The frequencies and titers of anti-β_1, β_2 and α_1-ARs are significantly higher in patients with chronic CRS, when compared to the heart failure group and the healthy control group. With the increasing serum creatinine, titers of these autoantibodies are gradually increased.

The pathogenesis of chronic CRS has remained obscure, but it is likely multifactorial, involving RAAS effects, NO and ROS imbalance, inflammatory response, SNS overactivity [12]. But the immunological mechanism of chronic CRS has not yet been reported.

There are biologically plausible mechanisms involving anti-β_1, β_2 and α_1-ARs leading to chronic CRS. The β_1 and β_2 ARs in the human heart couple to the G protein Gs, to activate adenylyl cyclase. Stimulation on both receptor subtypes increases the intracellular level of cAMP, which leads to phosphorylation of target proteins [13]. α_1-AR coupled predominantly via Gq, causes hydrolysis of membrane phospholipids via phospholipase C, to yield the second messengers inositol triphosphate and diacylglycerol, which leads to muscle contraction through mobilisation of intracellular Ca^{2+} [14] and activation of protein kinase C. Frequencies and titers of anti-β_1, β_2 and α_1-ARs are significantly increased in the chronic CRS group than in the heart failure group and healthy control group. Therefore we posit that there may be a relationship between the presence of anti-β_1, β_2 and α_1-ARs and the development of chronic CRS. Alternatively, it is plausible that chronic CRS triggers the production of anti-β_1, β_2 and α_1-ARs. Further studies are needed to delineate these two possible pathways.

Three kinds of autoantibodies closely related to each other were detected in the chronic CRS group. Approximately 43.3% (13/30) of chronic CRS patients have all three kinds of autoantibodies, which indicate that the autoimmune response in chronic CRS patients is multifaceted.

In conclusion, this pilot study has demonstrated for the first time that the presence and titers of anti-β_1, β_2 and α_1-ARs in patients with chronic CRS are all significantly increased compared with the heart failure group and healthy control group. With the increasing serum creatinine, titers of three autoantibodies are gradually increased. So, the correlation between the presence and titers of autoantibodies against adrenoreceptors and chronic CRS is very high. We posit that immunological mechanisms may be involved in the pathogenesis of chronic CRS. Further studies are needed to confirm these findings and dissect the underlying mechanisms for this novel observation.

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Disclosure of conflict of interest

None.

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