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Cardiac effects of long-term active immunization with the second extracellular loop of human β_1- and/or β_3-adrenoceptors in Lewis rats

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ABSTRACT

β_1- and β_3-adrenoceptor (AR) auto-antibodies were detected in patients with dilated cardiomyopathy. Many studies have shown that β_1-AR auto-antibodies with partial agonist-like effect play an important role in the pathogenesis of this disease. Moreover, a recent study carried out in our laboratory has shown that β_3-AR antibodies (β_3-ABs), produced in rats, were able to reduce cardiomyocyte contractility *via* β_3-AR activation. The aims of this study were (1) to investigate, in isolated cardiomyocytes from rabbit, the role of G_i proteins in the β_3-ABs-induced cardiac negative inotropy, (2) to determine whether β_3-ABs may exhibit β_3-AR antagonistic property which is characteristic of partial agonists, and (3) to determine whether long-term active immunization producing both β_1-ABs and/or β_3-ABs leads to the development of cardiac dysfunction in Lewis rats.

Lewis rats were immunized for 6 months with peptidic sequences corresponding to the second extracellular loop of human β_3-AR and/or β_1-AR. Agonistic effect of β_3-ABs was evaluated on electrically field-stimulated isolated cardiomyocytes from adult rabbit by measuring the cell shortening. Echocardiography and *ex vivo* isolated perfused heart studies were conducted on immunized rats. Finally, β-AR expression was quantified by immunofluorescence and RT-qPCR.

SR58611A (10 nM), a preferential β_3-AR agonist, and purified β_3-ABs (25 μg/ml) induced a decrease in cell shortening ($-39.71 \pm 4.9\%$ ($n = 10$) and $-17.06 \pm 3.9\%$ ($n = 10$) respectively). This effect was significantly inhibited when the cardiomyocytes were preincubated with pertussis toxin (0.3 μg/ml), a G_i protein inhibitor ($p < 0.05$). In addition, SR58611A-mediated negative inotropic effect was decreased when cardiomyocytes were preincubated with β_3-ABs ($p < 0.0001$). Echocardiography revealed a decrease in the fractional shortening and ejection fraction in rats immunized against β_1-AR and both β_1- and β_3-AR. However, the study on isolated heart showed a decrease of the isoproterenol-induced lusitropic and inotropic effects in the 3 groups of immunized rats. These systolic and diastolic dysfunctions are correlated with a decrease in the expression of β_1-ARs and an increase of β_3-ARs in rats immunized against the β_1-AR and an increase of both β_3-AR and β_1-AR in rats immunized against the β_3-AR. For the first time, these results showed that β_3-ABs had a β_3-AR partial agonist-like activity which might play a role in the pathogenesis of cardiac dysfunction.

Abbreviations: AB, antibody; AR, adrenoceptor; AAB, autoantibody; cAMP, cyclic adenosine monophosphate; DCM, dilated cardiomyopathy; DP, developed pressure; dP/dt, time derivatives of pressure; EDD, left ventricular end-diastolic diameter; EDV, left ventricular end-diastolic volume; EF, ejection fraction; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; ESD, left ventricular end-systolic diameter; ESV, left ventricular end-systolic volume; FS, fractional shortening; IgG, immunoglobulin; MFI, mean fluorescence intensity; NO, nitric oxide; OD, optical densities; PTX, pertussis toxin; SR58611A, [(RS)-N-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2R)-2(3-chlorophenyl)-2hydroethanamine hydrochloride].

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1. Introduction

Idiopathic dilated cardiomyopathy (DCM) is one of the main cause of severe heart failure in young adults. In 60–70% of cases, the etiology remains poorly understood and growing evidences suggest that the immunity system may play a key role in this disease [1].

β_1-Adrenoceptor (AR) auto-antibodies (AABs) were first detected by ELISA (enzyme-linked immunosorbent) in 26 to 60% of patients with DCM [2,3]. These β_1-AABs are directed against the second extracellular loop of human β_1-AR [4]. *In vitro* and *in vivo* studies have shown that β_1-AABs induce positive inotropic and chronotropic effects *via* the β_1-AR/adenylate cyclase/cAMP-dependent protein kinase-A pathway [5–8]. According to Magnusson et al. [7], these β_1-AABs share some properties of partial agonists. They can act as an agonist and activate weakly the β_1-ARs. On the contrary, they can act as an antagonist of the β_1-ARs and block them when the catecholamine levels are high. Furthermore, rat or rabbit immunization with the second extracellular loop of β_1-AR has been reported to be able to induce myocardial dysfunction that may lead to ventricle dilatation similar to that observed in patients [9,10]. This effect is considered to result from long-term overstimulation of the β_1-ARs by the β_1-AABs. In agreement with those observations, clinical studies have also found that β_1-AABs suppression by immunoabsorption improves cardiac function of patients with DCM [11,12], strengthening the possibility that β_1-AABs play an important pathophysiological role in the development of this disease.

More recently, circulating β_3-AABs directed against the second extracellular loop of the β_3-AR, have also been detected in 30% of sera from patients with heart failure [13]. A recent study carried out in our laboratory has shown that β_3-AR antibodies (β_3-ABs), produced in rats, were able to reduce cardiomyocyte contractility *via* β_3-AR activation [14]. Nevertheless, very few studies have been done to characterize these auto-antibodies and to evaluate their involvement in DCM.

In addition to β_1-AR, β_3-AR is also expressed in the human and animal ventricle myocardium. Its activation was described to induce a negative inotropic effect that involved G_i protein/nitric oxide (NO) pathway [15]. Several studies, conducted in failing or non-failing myocardium have reported that the β_1-ARs and β_3-ARs are cross-regulated by interactive compensatory mechanisms [16–19]. The opposed changes in the β_1-AR and β_3-AR-induced myocardial contraction in response to adrenergic stimulation seems to play a role in the worsening of the heart contractility. To the best of our knowledge, it is not known whether similar regulation may occur in response to β_1-AABs and β_3-AABs that could possess sympathomimetic-like properties. Therefore, the main objective of this study was to determine whether long-term active immunization producing both β_1- and/or β_3-ABs leads to the development of cardiac dysfunction in Lewis rats. Moreover, considering the β_3-ARs-mediated pertussis toxin (PTX)-sensitive effect in the heart [20] and the β_3-AR agonist-like activity of the β_3-ABs [14], we first investigated, in isolated cardiomyocytes from rabbit, the role of G_i proteins in the β_3-ABs-induced cardiac negative inotropy and whether β_3-ABs may exhibit β_3-AR antagonistic property.

2. Methods

2.1. Animals

Whole experimental project was validated by local ethics committee for animal experimentation (N°CEEA.2012.76) and conducted in accordance with "The guide for the care and use of laboratory animals" published by the National Institute of Health (NIH publication, eight edition, 2010). Nine week-old male Lewis rats from Janvier Labs (Le Genest Saint Isle, France) and New Zealand rabbits (2 kilograms) from Hypharm (Roussay, France) were used for this study. They were housed at constant temperature (22 ± 2 °C) and subjected to a cycle of dark/light 12:12 h, with standard chow and drinking water provided *ad libitum*.

2.2. Immunization protocol

Rats were immunized by subcutaneous injections of an antigen dissolved in 1 ml of a solution containing Na_2CO_3 (0.1 M) and β-mercapto-ethanol 1%, conjugated with Freund's adjuvant (V/V) monthly for 6 months. For that, rats were randomly divided into 4 groups. The first group ($n = 10$) was immunized with the antigen corresponding to the peptidic sequence of the second extracellular loop of human β_1-AR (residues 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; 1 mg/ml) synthesized by GeneCust (Dudelange, Luxembourg). The second group ($n = 10$) was immunized with the antigen corresponding to the peptidic sequence of the second extracellular loop of human β_3-AR (residues 176–200: R-V-G-A-D-A-E-A-Q-E-C-H-S-N-P-R-C-C-S-F-A-S-N-M-P; 2 mg/ml) (GeneCust, Dudelange, Luxembourg). The third group ($n = 10$) was immunized with both β_1-AR (1 mg/ml) and β_3-AR (2 mg/ml) peptides and the last group ($n = 10$) (adjuvant-treated) received only saline conjugated with Freund's adjuvant (0.5 ml).

2.3. β_1- and β_3-adrenergic receptor antibodies detection and purification

Sera were collected before the first immunization and after 2, 4 and 6 months of immunization. The evolution of β_1- and β_3-AB titers was followed by peptide-based ELISA.

For that, β_1-AR and β_3-AR peptides (10 μg/ml) used for the immunization were dissolved in BIC buffer (Na_2CO_3 0.1 mol/l; $NaHCO_3$ 0.1 mol/l; in distilled water; pH 9.6) and coated on a 96-well microplate (poly NUNC, Denmark) overnight at 4 °C. Serum dilutions (100 μl) from 1:400 to 1:51,200 in PBS-Tween 80-NaCl 0.5 mol/l, were used to react with the peptides for 1 h at 37 °C. After washing 3 times with PBS-Tween 80, 100 μl of donkey anti-rat immunoglobulin (IgG) antibody conjugated with horseradish peroxidase (1:50,000 dilution in PBS-Tween 80-NaCl 0.5 mol/l) (Jackson ImmunoResearch, USA) were added to the wells and incubated 1 h at 37 °C. After 3 washings, 100 μl of 3,3′, 5,5′-tetramethylbenzidine (Sigma–Aldrich) were incubated at 37 °C to detect the bound antibodies. The reaction was stopped after 20 min of incubation by adding 50 μl of sulphuric acid (0.1 mol/l). Optical densities (OD) were read at 450 nm in a microplate reader (TriStar, Berthold Technologies, Bad Wildbad, Germany). The antibody titers were defined by the OD values.

IgG fractions were purified from sera collected after 6 months of immunization using the Proteus Protein G kit (AbD Serotec, Colmar, France) in compliance with the manufacturer's instructions. Purified antibody concentrations were determined by the bicinchoninic acid protein assay (Uptima, Interchim, Montluçon, France).

2.4. Functional characterization of IgGs containing β_3-adrenergic receptor antibodies

The functionality of purified β_3-ABs was evaluated on ventricular cardiomyocytes isolated in healthy rabbit, which is known to express functional β_3-ARs [21]. Briefly, rabbits were anesthetized with pentobarbitone (54 mg/kg IV) and heparinized (2500 IU/kg IV). Cardiomyocytes were isolated by perfusion (7 ml/min) of heart mounted on a Langendorff apparatus with 1 mg/ml collagenase type II (Worthington, Lakewood, NI, USA) and 0.04 mg/ml protease