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E Montaudon, Laurence Dubreil, Valerie Lalanne, M. Vermot Des Roches, Gilles Toumaniantz, et al.. Cardiac effects of long-term active immunization with the second extracellular loop of human β 1- and/or β 3-adrenoceptors in Lewis rats. *Pharmacological Research*, 2015, 100, pp.210-219. 10.1016/j.phrs.2015.08.006 . hal-01208120

HAL Id: hal-01208120

<https://hal.science/hal-01208120>

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ARTICLE *in* PHARMACOLOGICAL RESEARCH · AUGUST 2015

Impact Factor: 4.41 · DOI: 10.1016/j.phrs.2015.08.006 · Source: PubMed

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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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ARTICLE INFO

Article history:

Received 6 March 2015

Received in revised form 28 June 2015

Accepted 7 August 2015

Available online 11 August 2015

Keywords:

Auto-antibodies

β -Adrenoceptor

Cardiac contractility

Dilated cardiomyopathy

Rats

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 μ g/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 μ g/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.

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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-[(2S)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2R)-2(3-chlorophenyl)-2-hydroethanamine 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 ($22 \pm 2^\circ\text{C}$) 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 $\mu\text{g/ml}$) 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

type XIV (Sigma–Aldrich, France) (30 μ M CaCl_2) for 20 min. After gentle manual stirring, cardiac cells were progressively exposed to increasing Ca^{2+} concentrations in 100% O_2 aerated Tyrode solution (NaCl 137 mM; KCl 5.4 mM; MgCl_2 1.2 mM; Na_2HPO_4 1.2 mM; HEPES 20 mM; D-glucose 10 mM; pH 7.4). Cardiomyocytes were plated on poly-L-lysine-coated 35 mm culture dishes and perfused at a flow rate of 3–4 ml/min with Tyrode solution (1.8 mM CaCl_2) at 37 °C. Cells were stimulated by an electric field (1 Hz) at 9 V and cell shortening was recorded using a video-imaging system (Coyote Bay Instruments, Manchester, NH, USA). Imaging analysis was performed using Matrox Inspector software (Coyote Bay Instruments, Manchester, NH, USA). Myocytes selected for data analysis presented clear striation, rod-shaped form and a stable diastolic length at baseline. Ten consecutive heart contractions were used for the analysis.

2.5. Echocardiography

At 6 months of immunization, rats were lightly anaesthetized with 1–1.5% isoflurane until the heart rate stabilized to 350–400 beats per minute. Transthoracic echocardiography was realized using a high frequency ultrasound imaging system (MyLab70 XVG, Esaote, Indianapolis, IN) with a linear 18 Mhz transducer. The focal length used was adjusted around 10 mm. To identify whether the immunization leads to the development of functional and structural cardiac dysfunctions, fraction shortening (FS), ejection fraction (EF), left ventricular end-systolic diameter (ESD), left ventricular end-diastolic diameter (EDD), left ventricular end-systolic volume (ESV) and left ventricular end-diastolic volume (EDV) were determined using M-Mode recordings in the short axis of the heart. ESV and EDV were calculated using the monoplan area-length method: $V = 8 \times A^2 / 3\pi L$. FS and EF were respectively calculated using the formulas $\text{FS} = [(\text{EDD} - \text{ESD}) / \text{EDD}] \times 100$ and $\text{EF} = [(\text{EDV} - \text{ESV}) / \text{EDV}] \times 100$. Three consecutive heart cycles were measured and the average used for analysis.

2.6. Ex vivo cardiac function

At 6 months of immunization, half of immunized rats were anaesthetized by intraperitoneal injection of pentobarbital (54 mg/kg) and heparinized (2500 IU/kg) ($n=5$, for each group). The heart was immediately removed and placed into ice-cold Krebs–Henseleit buffer (NaCl 118.3 mM; KCl 4.7 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2 mM; KH_2PO_4 1.2 mM; NaHCO_3 20 mM; EDTA 0.016 mM; Glucose 11.1 mM; CaCl_2 2.5 mM; pH 7.4). The aorta was rapidly cannulated and the heart retrogradely perfused by a non-recirculating-Langendorff technique at a constant flow of 10 ml/min in continuously gassed (95% O_2 /5% CO_2) prewarmed (37 °C) KH buffer. To determine left ventricular function, a latex balloon was inserted into the left ventricle through the mitral valve and filled until the left ventricular end-diastolic pressure reached a value of 5 mm Hg. An equilibration period of at least 30 min was required to ensure the stability of recorded parameters before any addition of molecule. For each immunized rat, systolic, diastolic, developed pressures and the heart rate were recorded initially and after the addition of (–)-isoproterenol hydrochloride (100 nM). Left ventricular developed pressure (DP) was determined as the difference between left ventricular systolic pressure and left ventricular end-diastolic pressure. Time derivatives of pressure were calculated electronically during contraction (dP/dT max) and relaxation (dP/dT min) using a Powerlab 8/30 Data Acquisition system and analyzed by LabChart® Pro software (V7, ADInstruments, France).

2.7. Real time quantitative RT-PCR

The other half of immunized rats ($n=5$, for each groups) were used to perform real time RT-qPCR to evaluate the effect of immunizations on the mRNA levels of β_1 -AR, β_2 -AR and β_3 -AR in the heart. Briefly, heart fragments were rapidly frozen in liquid nitrogen and stored at –80 °C. Frozen samples were dissolved in 1.5 ml of TRIzol® Reagent and ground to powder in a potter, according to manufacturer's instructions (Life Technologies, France). Total RNA were obtained after chloroform extraction and isopropanol precipitation and resuspended in 50 μ l of RNase-free water, heated at 58 °C and stored at –20 °C. The total RNA integrity was checked by agarose gel electrophoresis. Total RNA concentrations, 260/280 and 260/230 ratios were determined by spectrophotometry (Nanodrop 2000, Thermo Scientific, France).

To remove any contaminating genomic DNA, a DNase digestion step (DNase I recomb, Roche Diagnostics France, France) was carried out according to manufacturer's instructions. DNA-free total RNA were recovered in 20 μ l of RNase-free water. Absence of contaminating DNA fragments was again verified with a sensitive GAPDH PCR (primers in Table 1) and with agarose gel electrophoresis. DNA-free total RNA concentrations and DO ratios were then determined by spectrophotometry.

Each sample underwent the following reverse transcription step: 1 μ g of DNA-free total RNA was used in 20 μ l total of mix solution based on the Superscript IV Reverse-Transcriptase kit (Life Technologies, France), with both 1 μ l of Random Primers (3 μ g/ μ l) and 1 μ l of Oligo(dT)₂₀ Primer (50 μ M) for RT. Retrotranscribed RNA efficiency was further validated with a new GAPDH PCR and with agarose gel electrophoresis.

Real time PCR reactions were set in 15 μ l with 5X HOT FIREPol® EvaGreen® qPCR Mix (high ROX) (Solis BioDyne, Riia, Estonia), including either 5 μ l of diluted cDNA (1:6) and 1 μ l of each primers (10 μ M). After an initial denaturation/DNA polymerase activation step of 15 min at 95 °C, the PCR forty cycles consisted of 10 s at 95 °C denaturation step and of 1 min at 60 °C (for ADRB-1 and ADRB-2 primers) or 61 °C (for ADRB-3 and eNos primers) hybridization/elongation step. Melting curves were produced to confirm the presence of a single gene specific peak and the absence of primer dimers. The quantification of gene expression was based on the $2^{-\Delta\Delta\text{Ct}}$ method [27]. Briefly, the mean Ct value of the control house-keeping gene β -actin was subtracted from mean threshold cycle values of duplicates for each interest mRNA. The difference in the mean ΔCt values between adjuvant-treated rats and β -immunized rats allows the calculation of relative levels of induced or repressed expression of the interest gene.

2.8. Immunofluorescence and quantification of β -adrenergic receptor expression

Rat heart used for the RT-qPCR were also used to quantify β -AR expression in the heart ($n=5$, for each groups). For that, immediately after the excision, heart was transversally sectioned and the middle section was post-fixed by incubation in 4% phosphate-buffered paraformaldehyde (pH 7.4) for 4 h at 4 °C. After 3 washings with PBS solution, it was cryoprotected by overnight incubation in PBS containing 20% sucrose. Then, heart section was embedded in transverse position in Tissue Tek OCT medium and immediately frozen by immersion in liquid isopentane. Frozen sections (8 μ m thick) for immunohistochemical analysis were thawed and submerged for 5 min in acetone at 4 °C and nonspecific antigen binding was blocked by incubating sections for 1 h in a PBS solution containing 0.3% Triton 100X and 2% bovine serum albumin (Sigma) following by 1 h at 37 °C incubation in the blocking buffer with a rabbit polyclonal antibody against β_1 -AR (1:50, Santa Cruz Biotechnology Inc., CA, USA) or a goat polyclonal antibody against β_3 -AR

Table 1

Oligonucleotide primers used in the study. Oligonucleotide sequence orientations are indicated as forward (Fw) and reverse (rv).

Primer name	Accession number/publication	Sequence (5'– 3')	Amplicon size (bp)
ADRB-1-Fw	NM.012701	CTGCTACAACGACCCCAACTG	120
ADRB-1-Rv	[22]	AACACCCGGAGGTACACGAA	
ADRB-2-Fw	NM.012492	CTCCTTAACCTGGTGGGCTATG	127
ADRB-2-Rv	Our design	TCCCATAGGTTTTCGAAGAAGA	
ADRB-3-Fw	NM.013108	GCCGAGACTACAGACCATAACCA	79
ADRB-3-Rv	[23,24]	CATTACGAGGAGTCCCACTACCA	
eNos-Fw	NM.021838	AGCTGGATGAAGCCCGGTGAC	60
eNos-Rv	[25]	CCTCGTGGTAGCGTTGCTGA	
B-Act-Fw	NM.007393	TTGCTGACAGGATGCAGAAG	86
B-Act-Rv	Our design	GTACTTGGCTCAGGAGGAG	
GAPDH-Fw	AF106860	ACTGGCGTCTTACACCACCATGGAGAAGGCT	720
GAPDH-Rv	[26]	CTCCTTGGAGGCCATGTAGGCCATGAGGTC	

(1:100, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at 37 °C. After being washed in PBS, the sections were incubated with the secondary antibody, AlexaFluor 555-conjugated donkey anti-rabbit (1:300, Life Technologies, Saint Aubin, France) or donkey anti-goat (1:300, Life Technologies, Saint Aubin, France) during 1 h at room temperature. After being washed, the sections were stained with nuclear dye DRAQ5 (1/1000, BioStatus, Shepshed, United Kingdom) and mounted in Mowiol (Calbiochem, San Diego, CA, USA). The immunolabeled sections were scanned serially using the helium neon laser (543 nm) to observe Alexa fluor 555 (β -AR immunolabellings) and with a helium neon laser (633 nm) to observe DRAQ5 signals (nuclei). Each image was recorded in a separated channel (channel red for Alexa fluor 555 and channel blue for DRAQ5) and overlaid. Acquisitions were performed by using a confocal microscope (Nikon, C1, Champigny-sur-Marne, France). Image analysis were performed to evaluate β -AR expression level in the heart ventricle of immunized rats by using Fiji software. Mean Fluorescence Intensity (MFI) values for β -AR immunolabelling in each condition were acquired from 5 different fields of immunolabeled ventricle heart sections by section with 5 rats by conditions. Finally, analyses were performed at least on 550 ± 100 cardiac fibers by animal. The same threshold was used to measure the sum intensity fluorescence of β -AR immunolabelling in each section and the MFI was reported to total area of analyzed section.

2.9. Drugs

(-)-Isoproterenol hydrochloride, L-748337 hydrate, pertussis toxin from *Bordetella pertussis* were obtained from Sigma-Aldrich (France). SR58611A [(RS)-N-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronaphth-2-yl]-(2R)-2(3-chlorophenyl)-2hydroethanamine hydrochloride] was a generous gift from Sanofi group (France). All drugs were prepared in distilled water with the exception of SR58611A that was dissolved in ethanol. Sodium pentobarbital solution was purchased from CEVA Santé Animale (Libourne, France) and heparine choay from Sanofi Aventis (Paris, France).

2.10. Statistical analysis

Results were expressed as a mean $\pm$ SEM where n represents the number of rats or rabbits used for the study. Statistical differences in comparison with the adjuvant-treated rats were evaluated using unpaired Student's t test with Prism® software V.5. The conditions for the t -test were tested with a Kolmogorov–Smirnov' test for the Gaussian distribution and with an F -test for the homogeneity of variances. For the *ex vivo* study, differences were determined using nonparametric Mann-Whitney test and for the immunoflu-

orescence analysis using a univariate Student's t test (R software V3.0.1). A value for $p < 0.05$ was considered statistically significant.

3. Results

3.1. β -adrenoceptor antibody production and characterization

β_1 -ABs and β_3 -ABs were generated by immunizing rats for 6 months and their respective immunoreactivities were determined by ELISA. Two months after the first immunization, we observed a high production of specific β_1 -ABs in rats immunized with β_1 -AR or β_1 - and β_3 -AR peptides (Fig. 1). After the 6 months of immunization, β_1 -AB titers were still high and stable compared to the low level of production in antibody titers in β_3 -AR-immunized rats and adjuvant-treated rats (OD at 6 months: 2.09 ± 0.24 and 2.30 ± 0.21 for β_1 -AR and β_1 - and β_3 -AR-immunized rats respectively versus 0.07 ± 0.005 and 0.08 ± 0.03 for adjuvant-treated rats and β_3 -AR-immunized rats respectively). In the same way, we observed a high increase of β_3 -AB titers in both β_3 -AR-immunized rats and β_1 - and β_3 -AR-immunized rats after 6 months of immunization compared with the β_1 -AR-immunized rats and adjuvant-treated rats (OD at 6 months: 1.37 ± 0.26 and 2.28 ± 0.18 for β_3 -AR and β_1 - and β_3 -AR-immunized rats respectively versus 0.07 ± 0.006 and 0.14 ± 0.02 for adjuvant-treated rats and β_1 -AR-immunized rats respectively) (Fig. 1). The data highlighted antibody specificity for their antigen and the lack of cross-reactivity between the two subtypes of β -AR.

3.2. Negative inotropic effect of β_3 -adrenoceptor antibody

To determine whether β_3 -ABs exhibit an agonistic effect as it was already described for β_1 -ABs [8], we measured the effect of β_3 -AB perfusion on electrically field-stimulated cell shortening. β_3 -ABs induced a decrease of cell shortening ($-17.06 \pm 3.9\%$, $n = 10$) and this effect was significantly inhibited in presence of PTX ($0.3 \mu\text{g/ml}$), a G_i protein inhibitor, and L-748337 ($1 \mu\text{M}$), a selective β_3 -AR antagonist ($p < 0.05$) (Fig. 2a). SR58611A (10 nM), a preferential β_3 -AR agonist produced also a significant decrease of cell shortening which was stronger than that induced by β_3 -ABs ($-39.71 \pm 4.9\%$, $n = 10$ and $-3.88 \pm 3.4\%$, $n = 7$, respectively). This negative inotropic effect was not observed when cardiomyocytes were perfused with the antibodies from adjuvant-treated rats.

Then, assuming that β_3 -ABs behaved as partial β_3 -AR agonists, we tested their ability to exhibit β_3 -AR antagonistic properties. Incubation of rabbit cardiomyocytes for 2 h with β_3 -ABs ($25 \mu\text{g/ml}$) significantly reduced the SR58611A-induced negative inotropy ($-7.22 \pm 3.8\%$, $n = 4$) in comparison with antibodies from adjuvant-treated rats ($-27.47 \pm 8.23\%$, $n = 7$) (Fig. 2b).

Fig. 1. Curves showing the evolution of β_1 -adrenoceptor (β_1 -AR) (a) and β_3 -adrenoceptor (β_3 -AR) (b) antibody titers determined by ELISA at dilution 1/1600, in sera of adjuvant-treated rats (empty circles, $n=10$), β_1 -AR-immunized rats (empty squares, $n=10$), β_3 -AR-immunized rats (full circles, $n=10$) and β_1 -AR and β_3 -AR-immunized rats (full squares, $n=10$). Antibody titers are defined as mean $\pm$ SEM of optical density values, *** $p < 0.0001$ versus adjuvant-treated group determined by unpaired Student's t -test at the different times.

Fig. 2. Effects of β_3 -adrenoceptor (β_3 -AR) antibodies on rabbit isolated cardiomyocyte contractility. (a) Negative inotropic effect of β_3 -AR antibodies (25 μ g/ml) without (black bar, $n=11$) or with preincubation (2 h) in the presence of pertussis toxin (0.3 μ g/ml) ($n=4$) or L-748337 (1 μ M) ($n=4$), SR58611A (10 nM) (white bar, $nn=10$) and adjuvant antibodies (25 μ g/ml) (grey bar, $n=7$). (b) Negative inotropic effect of SR58611A (10 nM) with preincubation (2 h) in the presence of β_3 -AR antibodies (25 μ g/ml) (black bar, $n=6$) or adjuvant antibodies (25 μ g/ml) (grey bar, $n=7$). Values were represented as mean $\pm$ SEM, * $p < 0.05$, *** $p < 0.0001$ versus cells that were not preincubated, determined by unpaired Student's t -test.

3.3. Influence of immunizations on cardiac function—in vivo study

To evaluate whether the different immunizations were able to impact the cardiac contractility, we performed echocardiography. Data indicated that EF and FS were decreased in rats immunized during 6 months with β_1 -AR (EF: -3.7% and FS: -5.1% versus adjuvant-treated rats) or both $\beta_{1/3}$ -AR peptides (EF: -5.6% and FS: -7.8% versus adjuvant-treated rats) compared to adjuvant-treated rats (EF: 77.50 ± 0.47 ; FS: 47.53 ± 0.48) (Fig. 3). The EDD and ESD were not altered by the immunization in β_1 -AR-immunized rats while in $\beta_{1/3}$ -AR-immunized rats both EDD and ESD were increased (EDD: $+4.02\%$ and ESD: $+11.13\%$ versus adjuvant-treated rats) (Fig. 3). In contrast, immunization with only β_3 -AR peptide

did not alter the EF and FS but increased the EDD ($+2.73\%$ versus adjuvant-treated rats) (Fig. 3c). Moreover, systolic blood pressure was not modified during the 6 months of immunization in the 4 groups of rats (data not shown) suggesting that these observed effects on cardiac contractility were not due to changes in arterial pressure and did not impact blood pressure.

3.4. Influence of immunizations on cardiac function—ex vivo study

The influence of chronic immunization on left ventricular contractility was also evaluated *ex vivo* using a non-recirculating-Langendorff technique. For each group of rats, systolic, diastolic, developed pressures and the heart rate were recorded under

Fig. 3. Influence of immunization with peptides corresponding to the second extracellular loop of β_1 -adrenoceptor or β_3 -adrenoceptor or both β_1 - and β_3 -adrenoceptor and with adjuvant on cardiac function. (a) Ejection fraction (EF), (b) fractional shortening (FS), (c) left ventricular end-diastolic diameter (EDD), (d) left ventricular end-systolic diameter (ESD). Values were represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.001$ versus adjuvant-treated rats, $n = 5$ rats for each groups.

basal conditions and after the addition of isoproterenol (100 nM). The data showed that isoproterenol increased the heart rate, the maximal and minimal first derivative of change in pressure over time (dP/dt (max/min)) and the developed pressure of hearts from adjuvant-treated rats. These effects were decreased in hearts from rats immunized with β_1 -AR or β_3 -AR or $\beta_{1/3}$ -AR peptides (Fig. 4). Given that heart contractility at baseline was not altered by immunizations (data not shown), these findings revealed that immunizations with a β -AR peptide (β_1 -AR and/or β_3 -AR) decreased the isoproterenol-induced lusitropy (dP/dt min) and the inotropy parameters (dP/dt max and developed pressure). We showed that β_1 -AR and β_3 -AR-immunizations had a similar outcome on heart function.

3.5. Influence of immunizations on β -adrenoceptor mRNA and expression

RT-qPCR were carried out to determine whether chronic immunizations modified cardiac mRNA level of β -ARs. The data have shown that β_1 -AR-immunization induced a slight increase of β_1 -AR transcripts compared to adjuvant-treated rats whereas β_2 -AR, β_3 -AR and eNOS (endothelial NO synthase) transcripts seemed to not be changed. A similar transcription profile was also observed in β_1/β_3 -AR-immunized. The β_3 -AR-immunization induced a slight increase of β_1 -AR, β_3 -AR transcripts but no modification of the β_2 -AR and eNOS mRNA levels (Fig. 5). To determine the expression profile of the β_1 -AR and β_3 -AR proteins on heart, immunofluorescence labeling were carried out. First, the membrane localization of β -ARs was checked by performing a co-labeling of β -AR/Dystrophin B to confirm the antibody specificity. The co-labeling results showed that β_1 -ARs and β_3 -ARs were expressed at the cell membrane in heart of adjuvant-treated rats (Fig. 6). The data on expression β_1 -AR and β_3 -AR profile were generally in accordance with the observa-

tions on mRNA levels (Fig. 7). The β_1 -AR-immunization reduced β_1 -AR expression ($-11.89 \pm 9.74\%$; $n = 5$) but increased that of β_3 -ARs ($+51.42 \pm 15.93\%$; $n = 5$) whereas the β_3 -AR-immunization increased both β_1 -AR and β_3 -AR expressions ($+89.06 \pm 20.69\%$; $n = 5$ and $123.60 \pm 28.04\%$; $n = 5$, respectively).

4. Discussion

The present study clearly demonstrated that β_3 -ABs purified from β_3 -AR-immunized rats induced a β_3 -AR partial agonist-like activity on isolated rabbit cardiomyocytes. In addition, by *ex vivo* approach, we revealed that rat immunizations for 6 months producing functional β_1 -ABs and/or β_3 -ABs led to the development of both systolic and diastolic dysfunctions.

In this work, peptide-based ELISA method has allowed to test the specificity of the β_3 -ABs for the second extracellular loop of the β_3 -AR. Immunized rats have produced high concentration of β_1 -AR and β_3 -AR IgG-like ABs. In addition, β_3 -ABs did not cross-react with β_1 -ARs that share a high degree of structural and biochemical similarities with β_3 -ARs. According to Bornholz et al. [2], β_1 -AABs from patients with DCM preferentially recognize a native β_1 -AR conformation. Therefore, the peptide-based ELISA method seems not to be the most appropriate method to detect human β_1 -AABs. However, in this present work, the use of peptides to detect antibodies generated by immunization in rats was not an issue because we used the same peptides for both the immunization and the detection of antibodies.

We previously showed that β_1 -ABs produced by immunization of rats with β_1 -AR peptide induced a positive inotropic effect [8]. In order to test whether β_3 -ABs possess also biological activity, we performed inotropic study on cardiomyocytes isolated from adult healthy rabbit. The β_3 -ABs-induced pertussis toxin-sensitive effect strongly suggests that the β_3 -ABs are endowed with negative inotropic action on cardiomyocytes that involves β_3 -AR/ G_i protein

Fig. 4. Cardiac responses to isoproterenol (100 nM) in adjuvant-treated rats ($n=5$) and rats immunized with peptides corresponding to the second extracellular loop of β_1 -adrenoceptor (AR) ($n=5$) or β_3 -AR ($n=5$) or both β_1 -AR and β_3 -AR ($n=5$). Heart rate (HR), time derivatives of pressure during contraction (dP/dt max) and relaxation (dP/dt min) and developed pressure (DP) were represented as mean $\pm$ SEM of percentage increase from baseline with set at 100%, * $p < 0.05$ versus adjuvant-treated rats, determined by nonparametric Mann–Whitney test.

Fig. 5. Comparative analysis of the mRNA levels of β_1 -adrenoceptor (AR), β_2 -AR, β_3 -AR and endothelial nitric oxide synthase (eNOS) in heart from adjuvant-treated rats (grey bars), β_1 -AR-immunized rats (black bars), β_3 -AR-immunized rats (white bars) and both β_1 - and β_3 -AR-immunized rats (stripped bars). Values were expressed as $2^{-\Delta\Delta C_t}$ using β -actin as a housekeeping gene and were represented as mean $\pm$ SEM.

pathway. In addition, the decrease of SR58611-induced negative inotropy after the incubation of cardiomyocyte in presence of β_3 -ABs indicates that the β_3 -ABs can also antagonize the β_3 -ARs. Taken together, these results are in favor of a partial agonist-like activity of the β_3 -ABs on heart.

The immunization protocol used to produce specific antibodies behaving like AABs has been already applied to study the cardiostimulating effects of β_1 -AABs in rat and rabbit [8,28]. Our findings agree with a recent study showing that β_3 -AABs purified from patients with DCM induced negative inotropic and negative chronotropic effects on rat neonatal cardiomyocytes [29]. Therefore, it might be argued that β_3 -ABs as well as β_1 -ABs would not

Fig. 6. Immunofluorescent co-labeling of β_1 -adrenoceptors (ARs) or β_3 -ARs and dystrophin B in heart from adjuvant-treated rats.

Fig. 7. Influence of immunization with peptides corresponding to the second extracellular loop of β_1 -adrenoceptor (AR) ($n = 5$) or β_3 -AR ($n = 5$) on β_1 -AR (white bars) and β_3 -AR (black bars) expressions in heart compared to adjuvant-treated rats. Values were represented as mean $\pm$ SEM of percentage increase of fluorescence from adjuvant-treated rats. Values were represented as mean $\pm$ SEM, ** $p < 0.001$, determined by univariated Student's t -test.

be just a biomarker, as stated by Miao et al. [30], but would have a functional role which needs more thorough investigation.

To evaluate whether circulating β_1 -ABs and β_3 -ABs having agonistic properties were able to impact the myocardial function, rats were immunized during 6 months with β_1 -AR and/or β_3 -AR peptides.

As it was already described [31,32], our results showed a slight but a significant decrease of fractional shortening and ejection fraction in rats immunized 6 months with the β_1 -AR peptide without modifications of systolic arterial pressure, assessed by tail cuff method. These observations were concordant with previous studies showing that left ventricle dysfunctions appeared after 6 months of immunization against β_1 -AR [9,10]. However, in our study, the inability of β_1 -ABs to induce the development of left ventricle dilatation (*i.e.*, no modification of end-systolic and -diastolic diameters) is likely linked to the fact that 6 months of immunization is not sufficient to observe noticeable alteration in myocardial contractility. In accordance with echocardiographic results, our *ex vivo* findings also revealed both systolic and diastolic dysfunctions in rats immunized with the β_1 -AR or both β_1 - and β_3 -AR peptides. These dysfunctions were characterized by a reduction of isoproterenol-induced inotropy and lusitropy that likely involved an impairment of global β -AR reactivity (*i.e.*, modification in β -AR expression or β -ARs-linked mechanisms). In line with this hypothesis, the quantification of β -AR expression by immunofluorescence revealed a downregulation of β_1 -ARs and an up-regulation of β_3 -ARs in β_1 -AR-immunized rats when compared with the adjuvant-treated rats in the left ventricle. This pattern of regulation was already reported to be involved in the alteration of myocardial contractility and was similar to that observed in patients with DCM [16,17]. Surprisingly, the β_1 -AR mRNA was not decreased and the β_3 -AR mRNA was not significantly increased in β_1 -AR-immunized rats. The reason of this discrepancy is not readily apparent in our study, but the change in the post-transcriptional or in the post-translational mechanisms could explain the lack of correlation between mRNA and protein expressions which remains to be elucidated.

One possible explanation to account for the cross-regulation of the β_1 -ARs and the β_3 -ARs in our study is that β_1 -ABs, by their chronic β_1 -AR agonistic action following immunization, could desensitize β_1 -ARs and could upregulate β_3 -ARs as a compensatory mechanism. Some studies have described the loss of β_1 -ARs in response to β_1 -AABs [33] or the existence of cross-regulation between β_1 -ARs and β_3 -ARs in the heart when β_1 -ARs are chronically stimulated [34]. The upregulation of β_3 -ARs is considered useful in the first stage of the heart failure because of its protective effect by antagonizing the β_1 -AR overstimulation.

Similarly to β_1 -AR-immunized rats, *ex vivo* study has revealed the development of both diastolic and systolic dysfunctions in β_3 -AR-immunized rats, which were characterized by a decrease of isoproterenol-induced inotropy and lusitropy. Thereby, we showed in this study that the production of cardio-stimulatory β_1 -ABs or cardio-inhibitory β_3 -ABs by immunizing rats with β_1 -AR or β_3 -AR peptide had a similar outcome on heart function. In rats immunized with β_3 -AR peptide, the immunofluorescence assay and RT-qPCR have highlighted an upregulation of β_3 -AR expression and mRNA in the left ventricle. Thus, it is not unreasonable to postulate that isoproterenol-mediated negative inotropic effect *via* β_3 -AR activation could have contributed to the impairment of cardiac function in β_3 -AR-immunized rats. Ideally the effect of isoproterenol in the presence of β_3 -AR antagonist should have been performed to accurately confirm the role of the β_3 -ARs in the impairment of isoproterenol-induced inotropy. In our study, since eNOS has been shown to play a role in the β_3 -ARs-mediated negative inotropy [15], we tested the hypothesis that its increased

expression through a larger production of NO could explain the depressed contractility observed in β_3 -AR-immunized rats. Nevertheless, the lack of change of eNOS mRNA in these rats argues against a role of the G_i protein/NO pathway to account for the reduced response to isoproterenol and supports the view of the existence of alternative mechanisms (*i.e.*, reduced cAMP level *via* G_i proteins) that need further investigation.

Although the present study has not specifically addressed the potential mechanism involved in the upregulation of ventricular β_3 -ARs, sustained activation of β_3 -ARs by β_3 -ABs following long-term immunization could be considered as one possible explanation of this pattern of regulation. In accordance with this contention, recent studies have shown that stimulation of the β_3 -ARs induced an increase of the β_3 -AR expression [35,36]. In addition, it was shown that an upregulation of β_3 -ARs was able to alter contractile response to isoproterenol in mice [37] and in human failing myocardium [16]. Nevertheless, those findings do not agree with the previous report [29,38] showing the protective role of β_3 -AABs against cardiac dysfunction, and do not support the point of view that β_3 -AR upregulation may be regarded as cardioprotective mechanism that may be developed to prevent cardiomyocyte damage.

More interestingly, the dysfunctions observed *ex vivo* in rats immunized against the β_3 -AR were not detected by our echocardiographic measurements. We showed that 6 months of immunization with β_3 -AR peptide did not modify the fractional shortening and ejection fraction. The lack of correlation between *ex vivo* and echocardiographic parameters has already been reported [39]. In our study, the reason of this discrepancy is not clear, but it may be explained by the intervention of compensatory neuro-hormonal mechanisms that might operate *in vivo* through β_1 -ARs to maintain myocardial contractility. This emphasizes the need to take into account the physiological context in the interpreting and the evaluation of left ventricular function. Moreover, based on our results, we cannot rule out the hypothesis that under *in vivo* conditions, circulating β_3 -ABs could have exhibited a β_3 -antagonistic property in response to endogenous catecholamines that might be responsible for undetectable cardiac dysfunction implying β_3 -AR component. However, whether prolonged immunization period with β_3 -AR peptide could induce impairment of echocardiographic contractile parameters remains to be determined.

There are some limitations of this study. First, concerning the functional study of β_3 -ABs, despite that rabbit cardiomyocytes constitute a relevant approach to assess *in vitro* effects of β_3 -ABs, translation to *in vivo* or to the clinical setting should only be made with caution. The β_3 -ABs produced by immunization in rats could have a different action than that of β_3 -AABs from patients with DCM which needs further investigation in the future. Second, concerning the influence of immunization, the immunization time of 6 months has been selected because Zuo et al. [9] have reported that the left ventricular dilation appeared from 5 months of immunization with β_1 -AR peptide. However, in view of our results, it seems that the putative effect of β_3 -ABs on cardiac function is a more slowly process and hence, the immunization time would need to be increased in the future. Third, in contrast to the *ex vivo* experiments, our echocardiographic approach failed to find any evidence of cardiac dysfunction in β_3 -AR-immunized rats. This suggests that measurements of fractional shortening and ejection fraction would not be always suitable for the evaluating of heart contractility under basal *in vivo* conditions. Therefore, future experiments using exogenous inotropic agents (*i.e.*, isoproterenol) will be required to accurately quantify the change in the myocardial contractility that may occur in immunized rats.

5. Conclusions

The results of the present study showed for the first time that β_3 -ABs induced a β_3 -AR partial agonist-like activity involving a role of G_i proteins in isolated rabbit cardiomyocytes. In addition, we show that immunizations for 6 months producing functional β_1 -ABs or β_3 -ABs led to the development of systolic and diastolic dysfunctions in heart by remodeling the β_1 -AR/ β_3 -AR ratio in the left ventricle.

Conflicts of interest

None.

Acknowledgements

The authors would like to thank Chantal Thorin for her help concerning the statistical analysis and Mireille Ledevin and Sonia Becavin for the technical assistance.

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