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Autoantibody against Cardiac β_1-Adrenoceptor Induces Apoptosis in Cultured Neonatal Rat Cardiomyocytes

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Abstract To clarify whether apoptosis is involved in the injury processes induced by autoantibody against cardiac β_1-adrenoceptor, we investigated the biological and apoptotic effects of antibodies on cultured neonatal rat cardiomyocytes. Wistar rats were immunized with peptides corresponding to the second extracellular loop of the β_1-adrenoceptor to induce the production of anti-β_1-adrenoceptor antibodies in the sera. Immunoglobulin (Ig) G in the sera was detected using synthetic antigen enzyme-linked immunosorbent assay and purified using the diethylaminoethyl cellulose ion exchange technique. Apoptosis of cardiomyocytes was evaluated using agarose gel electrophoresis and flow cytometry. Our results showed that the positive serum IgG greatly increased the beating rates of cardiomyocytes and showed an "agonist-like" activity. Furthermore, positive serum IgG induced cardiomyocyte apoptosis after treatment with β_1-adrenoceptor overstimulation for 48 h. The effects of monoclonal antibody against β_1-adrenoceptor were also found to be similar to those of positive serum IgG. It was suggested that the autoantibody could induce cardiomyocyte apoptosis by excessive stimulation of β_1-adrenoceptor.

Key words β_1-adrenoceptor; rat cardiomyocyte; autoantibody; apoptosis

In recent years, autoantibodies against cardiac β_1-adrenoceptors have been found in approximately 30% of patients suffering from dilated cardiomyopathy (DCM) by enzyme-linked immunosorbent assay [1–3]. The high level of β_1-adrenoceptor autoantibodies is related to poor left ventricular ejection fraction in patients [4–7]. *In vivo* experiments also showed that active immunization of rats stimulated strong production of anti-β_1-adrenoceptor antibodies in the sera, which had a long-term effect on the heart and induced remarkable histopathological changes and cardiac dysfunction [8,9]. In addition, it has been demonstrated that these autoantibodies could specifically recognize the functional epitope of the second extracellular loop of the corresponding receptors and display various agonist-like activities without desensitization [1,2]. All of these results indicated that anti-β_1-adrenoceptor antibodies might be involved in the pathologic processes of heart failure.

It is known that apoptosis is one of the most common causes of cell loss in humans and animals, and is characterized by nuclear and cellular fragmentation. There is increasing evidence that apoptosis is mediated even in a low-grade manner. Moreover, inhibiting apoptosis can successfully prevent or attenuate heart failure [10]. However, the mechanisms of the pathogenic effects are still unclear.

Previous reports indicated that overdriving of β_1-adrenoceptor could induce apoptosis in neonatal and adult cardiomyocytes [11]. Furthermore, β_1-adrenoceptor antibodies can produce a positive inotropic response as a partial agonist in isolated cardiomyocytes [12–14]. The apoptotic effect of the antibody against β_1-adrenoceptor on cardiomyocytes needs to be investigated.

In this study, we assessed the biological and apoptotic effects of anti-β_1-adrenoceptor autoantibody on cultured neonatal cardiomyocytes by immunizing rats using a peptide corresponding to the second extracellular loop of β_1-adrenoceptor as the immunogen.

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Materials and Methods

Synthetic peptide and anti-β_1-adrenoceptor monoclonal antibody

The free peptide (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), corresponding to the sequence of the second extracellular loop of the human β_1-adrenoceptor, was synthesized by the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The peptide was analyzed by high performance liquid chromatography on a Vydac C-18 column, and amino acids were analyzed by an automated amino acid analyzer (Beckman, Palo Alto, USA).

Monoclonal antibody M16 was a kind gift from Prof. Yvonne MAGNUSSON (Wallenberg Laboratory, Goteborg University, Goteborg, Sweden). The monoclonal antibody was obtained by immunizing BALB/c mice with the synthesized free peptide H26R.

Immunization

Twelve healthy Wistar rats (180–220 g) were divided into two groups: the control group (n=6), and the free peptide immunized group (n=6). Rats in the latter group were immunized using 0.4 μg/g body weight of the corresponding peptide (free peptide, 100 μg/ml in saline) which was emulsified in 1 ml of complete Freund's adjuvant (Sigma, St. Louis, USA), and injected subcutaneously at multiple points. Fourteen days later, a booster injection of a mixture of 0.4 μg/g body weight of the corresponding peptide (free peptide, 100 μg/ml in saline) in 1 ml of incomplete Freund's adjuvant (Sigma) was injected subcutaneously at one point. The control rats were injected using saline instead of the antigen peptide following the same procedure as the immunized group. The blood samples of all rats were collected for detection of serum anti-β_1-adrenoceptor antibodies 1 d before immunization or 14 d after the booster injection. Rats were also bled through cardiac puncture and the sera were collected for future use.

The immunoglobulin (Ig) G was prepared from collected sera by graded salt precipitation in 35%–40% $(NH_4)_2SO_4$ solution and dialyzed extensively against phosphate-buffered saline (PBS). After salt depletion by Sephadex G25 chromatography, the desalted protein was further purified by diethylaminoethyl cellulose ion exchange chromatography. The purity of IgG was assessed by polyacrylamide gel electrophoresis.

Enzyme-linked immunosorbent assay

The synthesized peptide was dissolved in a 100 mM Na_2CO_3 solution (pH 11.0) to a final concentration of 10 μg/ml. The peptide (0.5 μg) was coated on 96-well plates (Nunc, Kastrup, Denmark) overnight at 4 ºC. The wells were then blocked with PMT [PBS supplemented with 3% (*W*/*V*) skimmed milk, 0.1% (*V*/*V*) Tween 20 and 0.01% (*W*/*V*) Thimerosal (Sigma)] for 1 h at 37 ºC. After washing the wells three times using PMT, 50 μl of serum with serial dilutions was added to each well and incubated for 1 h at 37 ºC. The wells were washed three times using PMT, and an affinity-purified biotinylated anti-rat IgG/BIO (Beijing Zhongshan Biotechnology, Beijing, China) was added at 1:1000 dilution. The plates were incubated for 1 h at 37 ºC, then washed and incubated with PMT containing 1 μg/ml streptavidin-peroxidase (Sigma) for 1 h. Then 2 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) was added as the substrate. Half an hour later, absorbance values were detected at 405 nm using a microplate reader (Molecular Devices, Menlo Park, USA). Positive reaction in the sera against the peptide was reported by Zhi *et al.* [15].

Culture of neonatal cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes were prepared according to the method originally described by Simpin and Savion [16] with minor modifications. Positive serum IgG for autoantibodies against β_1-adrenoceptors, negative control IgG, monoclonal antibody for β_1-adrenoceptor, or isoprenaline was used to treat the cells. After 30 min treatment, the number of beating single cardiomyocytes or clusters of synchronously contracting cardiomyocytes was counted and compared with that of pre-treatment. The basal beating rate was 110±20 beats per minute (b.p.m.). After 48 h of antibody or isoprenaline treatment, the cardiomyocytes were collected and cell apoptosis was detected.

DNA fragmentation (DNA ladder) assay

The protocol of Moore and Matlashewski [17] was used to isolate fragmented DNA. The experiment was normalized using equal volumes of cultures for each group. In brief, cardiomyocytes (approximately 3.0×10^6) from each treated sample were washed with PBS and the pellets were homogenized with 1 ml of lysis buffer (10 mM Tris, pH 7.4, 5 mM EDTA, 1% sodium dodecylsulfate, 0.3 M NaCl) for 20 min on ice. After centrifugation (11,000 *g*) for 20 min at 4 ºC, the supernatants containing fragmented DNA were collected and digested using 20 μg/ml RNase A

at 37 ºC for 1 h, then using 0.1 mg/ml proteinase K at 56 ºC for 3 h. The fragmented DNA was extracted using phenol and chloroform. The aqueous phase was transferred into a new Eppendorf tube and mixed with two volumes of ice-cold ethanol to precipitate DNA at –20 ºC overnight. The supernatant was discarded after centrifugation at 15,000 *g* for 20 min at 4 ºC. DNA pellets were washed with 80% ethanol and dissolved in TE buffer (pH 7.6). The entire sample was electrophoresed on 1.5% agarose gel for 1 h at 85 V. Then the gel was examined and photographed by a Gel documentation system (Ultra Violet Products, Cambridge, UK).

Flow cytometry assay

Cell apoptosis was estimated by flow cytometry assay as described previously [18]. Briefly, 1.0×10^6 cells were harvested, washed twice with cold PBS (pH 7.4) and fixed in 70% ice-cold ethanol. After centrifugation, washing with PBS and re-centrifugation, the cells were suspended in 1 ml of DNA staining solution (PBS containing 10 μg/ml RNase A and 50 μg/ml propidium iodide) and incubated for 30 min at room temperature in the dark. Then at least 1.0×10^5 cells from each sample were examined, and the percentage of apoptotic cells was calculated using CellQuest and modifit software packages (Becton Dickinson, San Jose, USA).

Statistical analysis

Results are expressed as the mean±standard deviation. The average of the antibody titer is expressed as the geometric mean value. Student's paired or non-paired *t*-test and ANOVA were done with SPSS version 10.0 software (SPSS, Chicago, USA). $P<0.05$ was considered as the significant difference.

Results

Antibody production and titers

Fourteen days after the first immunization, the titer in the sera was less than 1:10. However, the antibody titer in the immunized rats was increased to 1:(142.5±6.0) ($P<0.01$ compared with the titer lower than 1:10 in the control group) after the second immunization. It indicated that the autoantibody against the corresponding receptor was produced in great quantity after injection of β_1-adrenoceptor antigen peptide twice *in vivo* (**Table 1**). The autoantibody was nearly not detected in the saline control group.

Table 1 Titers of autoantibody against β_1-adrenoceptor in the sera of control and immunized rat groups

Group	*n*	Titer	
		2 weeks	4 weeks
Control	6	<1:10	<1:10
Immunized group	6	<1:10	1:(142.5±6.0)*

*$P<0.05$ versus control.

Chronotropic effects on cultured cardiomyocytes

Spontaneously beating rates of rat neonatal cardiomyocytes were used to assess the chronotropic effects of the antibodies. The data showed that the negative serum IgG control had no effect on beating rates, but the positive serum IgG for β_1-adrenoceptor, monoclonal antibody for β_1-adrenoceptor and β_1-adrenoceptor agonist isoprenaline significantly increased the beating rates of cardiomyocytes. The positive serum IgG at the concentrations of 0.3, 0.6 and 1.2 μM increased the rates to 17.2±7.1, 22.6±6.3 and 30.4±11.9 b.p.m., respectively. All of these data were statistically significant compared with those of the negative serum IgG group at the corresponding concentrations. Monoclonal antibody at 10, 50 and 100 nM increased the rate to 20.4±6.6, 24.8±8.6 and 35.6±13.6 b.p.m., respectively. Isoprenaline at a concentration of 10 μM increased the rates to 51.0±19.4 b.p.m.. The data in the latter two groups were also statistically significant compared with those of pre-treatment rats (**Table 2** and **Fig. 1**).

DNA ladder production by autoantibodies against β_1-adrenoceptor and isoprenaline

The positive serum IgG, monoclonal antibody and isoprenaline were added into cardiomyocyte culture media. After incubation for 48 h, all of the cultured neonatal cardiomyocytes showed characteristic DNA fragmentation, with a ladder of the internucleosomal DNA bands representing integer multiples of the internucleosomal DNA length (approximately 180 bp), but DNA ladders were not detected in the negative serum IgG group or the control group without any treatment (**Figs. 2**–**4**). This suggested that the overdriving of β_1-adrenoceptor induced cardiomyocyte apoptosis.

Apoptosis detection by flow cytometry

The effects of positive serum IgG against β_1-adrenoceptor (**Figs. 5** and **6**) and monoclonal antibody (**Figs. 7** and **8**) were examined at various concentrations by flow cytometry. Treatment with the antibodies resulted in a concentration-dependent increase in the apoptotic popula-

Table 2 Positive chronotropic effects of the positive serum IgG, monoclonal antibody, and isoprenaline on the beating rate of cultured neonatal rat cardiomyocytes

	Concentration	Beating rate (b.p.m.)		Beating rate increase
		Pre-treatment	Post-treatment	(b.p.m.)
Negative control	0.3 μM	110.2±19.3	109.2±15.6	5.0±4.0
	0.6 μM	108.0±22.0	108.0±23.2	3.0±1.7
	1.2 μM	118.0±17.7	118.0±19.7	5.2±3.3
Positive serum IgG	0.3 μM	110.8±21.2	128.0±20.5	17.2±7.1*
	0.6 μM	99.6±25.0	122.2±25.1	22.6±6.3*
	1.2 μM	111.0±16.4	135.6±12.0	30.4±11.9*
Monoclonal antibody	10.0 nM	117.0±21.8	137.6±21.2	20.4±6.6**
	50.0 nM	106.8±20.7	131.6±13.7	24.8±8.6**
	100.0 nM	108.2±16.1	143.8±12.5	35.6±13.6**
Isoprenaline	10.0 μM	113.6±28.4	164.6±27.2	51.0±19.4**

*P<0.05 versus corresponding group in negative control; **P<0.01 post-treatment group versus pre-treatment group. b.p.m., beats per minute.

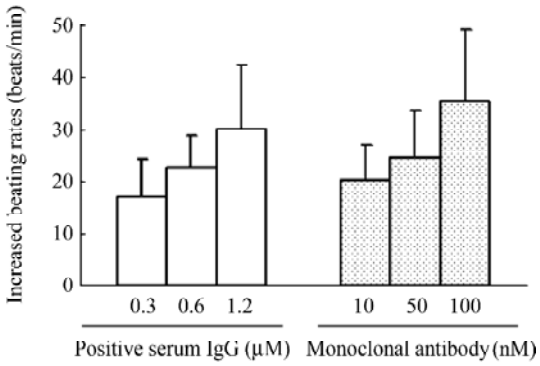

Fig. 1 Chronotropic effects of positive serum IgG and monoclonal antibody against β_1-adrenoceptor on the beating rate of cultured neonatal rat cardiomyocytes

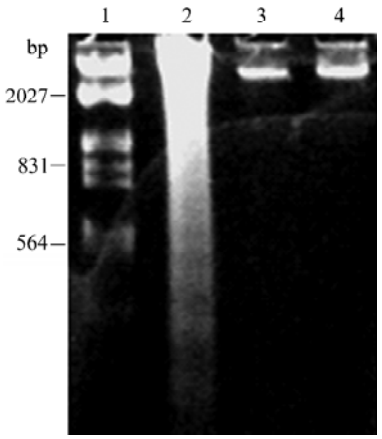

Fig. 3 Detection of apoptotic DNA ladder in cultured neonatal rat cardiomyocytes after monoclonal antibody treatment for 48 h

1, marker; 2, monoclonal antibody (100 nM); 3, negative control; 4, control.

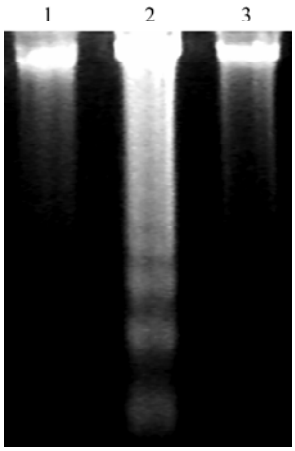

Fig. 2 Detection of apoptotic DNA ladder in cultured neonatal rat cardiomyocytes after immunoglobulin (Ig) G positive sera treatment for 48 h

1, negative control; 2, positive serum IgG (1.2 μM); 3, control.

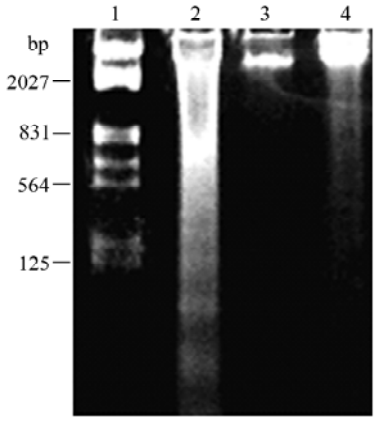

Fig. 4 Detection of apoptotic DNA ladder in cultured neonatal rat cardiomyocytes after isoprenaline treatment for 48 h

1, marker; 2, isoprenaline (10 μM); 3, control; 4, negative control.

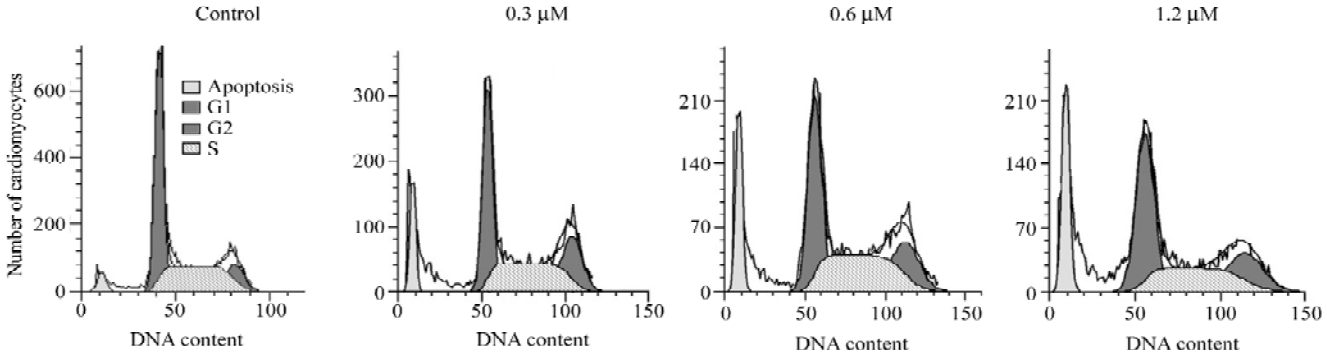

Fig. 5 Flow cytometry analysis of cardiomyocyte apoptosis after incubation with positive serum IgG

Neonatal rat cardiomyocytes were treated with positive serum IgG at different concentrations.

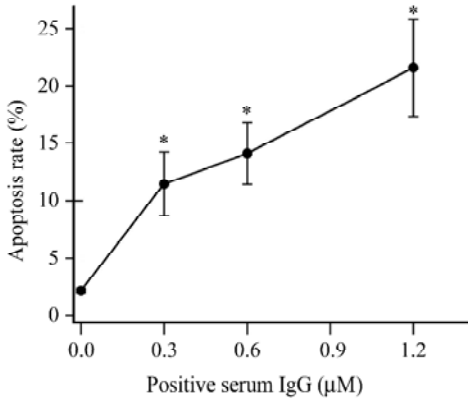

Fig. 6 Cardiomyocyte apoptosis after incubation with positive serum IgG

*P<0.01 versus control.

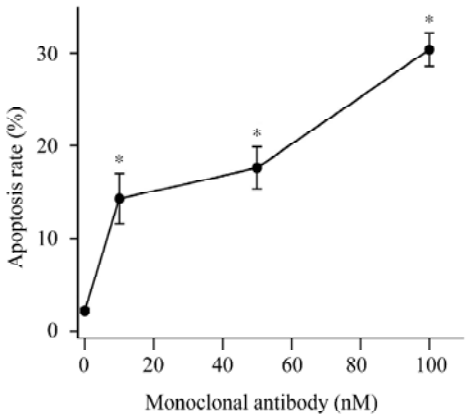

Fig. 8 Cardiomyocyte apoptosis after incubation with monoclonal antibody

*P<0.01 versus control.

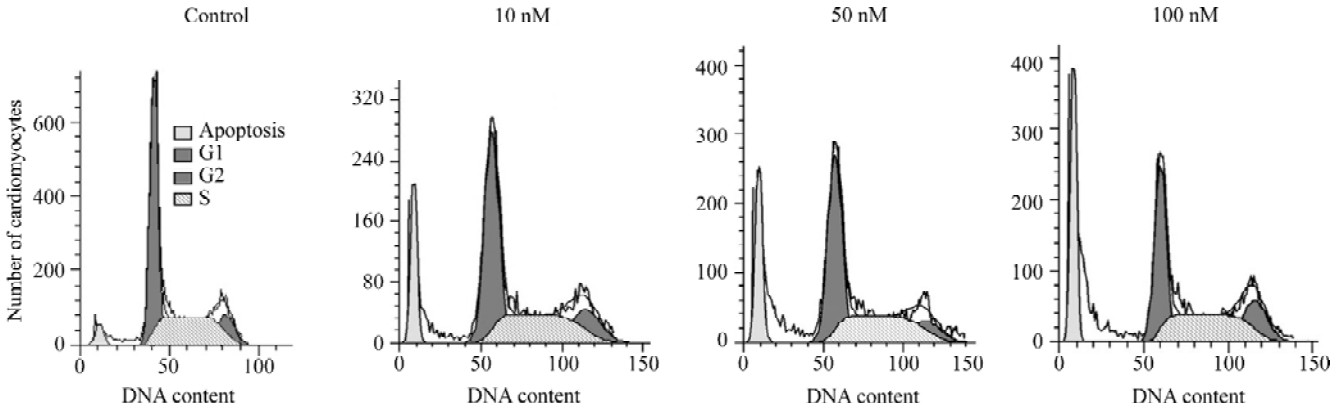

Fig. 7 Flow cytometry analysis of cardiomyocyte apoptosis after incubation with monoclonal antibody

Neonatal rat cardiomyocytes were treated with monoclonal antibody at different concentrations.

tions (**Table 3**). The apoptotic effects of the positive serum IgG group were increased significantly to 11.5%±2.8%, 14.1%±2.7% and 21.6%±4.2% at the concentrations of 0.3 μM, 0.6 μM and 1.2 μM, respectively. In the monoclonal antibody group, the percentages of the apoptotic cells were 14.3%±2.7% at 10 nM, 17.6%±2.3% at 50 nM and 30.4%±1.8% at 100 nM. All of these data were significantly different compared with that of the corresponding control (P<0.01).

Table 3 Percentages of apoptotic cardiomyocytes induced by positive serum IgG and monoclonal antibody against β_1-adrenoceptor detected by flow cytometry assay

Positive serum IgG		Monoclonal antibody	
Dose (μM)	Rate (%) [a]	Dose (nM)	Rate (%) [a]
Control	2.17±0.24	Control	2.17±0.24
0.3	11.48±2.76*	10	14.32±2.71*
0.6	14.10±2.65*	50	17.61±2.25*
1.2	21.57±4.22*	100	30.39±1.81*

[a] the apotosis rate. *P<0.01 versus control.

Discussion

Increasing evidence suggests that autoimmune response is involved in the pathogenesis of a number of cardiovascular diseases, including DCM. Among them, the biological, functional and pathogenic properties of anticardiac receptor antibodies have been extensively investigated. It was recently reported that autoantibody against cardiac troponin I could cause DCM in normal mice [19]. Jahns *et al.* also revealed these antibodies against various synthetic receptor peptides in 51% of patients suffering from DCM, but only the subgroup against the second extracellular loop could bind and stimulate human β_1-adrenoceptor of the cell membrane [4]. Approximately 31% of patients with DCM develop autoantibody against this epitope. Much research has indicated that autoantibody against β_1-adrenoceptor also played an important role in the pathogenesis of DCM, such as cardiac morphologic changes, deposition of collagen and obvious functional impairment [7,20,21]. An earlier study found that active immunization with the synthetic peptide corresponding to the second extracellular loop of the human cardiac β_1-adrenoceptor in rabbits or rats could induce a remarkable production of anti-receptor peptide antibody, which had the same biological and immunological properties as those of autoantibody in the sera of patients with DCM [13]. Therefore, in the present study, we used a synthetic peptide corresponding to the second extracellular loop of human β_1-adrenoceptor as the antigen to immunize rats to induce the generation of autoantibody, and then investigate the role of anti-β_1-adrenoceptor antibody on cultured neonatal cardiomyocytes.

The apoptosis in hearts of patients with heart failure has increased the possibility that apoptosis contributes to the pathophysiology of myocardial failure. Pharmacologic studies of cardiomyocytes *in vitro* demonstrate that β_1-adrenoceptor can stimulate apoptosis. It was reported that overexpression of β_1-adrenoceptor was associated with myocyte apoptosis and the development of DCM in transgenic mice [22,23]. Here, we used cultured neonatal cardiomyocytes as a model to study whether overstimulation of β_1-adrenoceptor by autoantibody could induce apoptosis. We chose neonatal cardiomyocytes as a model because the cultured technique of neonatal rat cardiomyocytes is well developed and accepted, and reliable. This study showed that positive serum IgG against the second extracellular loop of β_1-adrenoceptor could clearly increase the beating rates and induce apoptosis in cultured neonatal cardiomyocytes in a dose-dependent manner.

It is known that positive serum IgG is a polyclonal antibody, which recognizes different independent epitopes on the antigen; monoclonal antibody is identical, but recognizes only one specific epitope and has a defined specificity for the antigen. To verify that the agonist-like activity and the apoptotic effects of the positive serum IgG were produced by the second extracellular loop of β_1-adrenoceptor, we also made a comparative study of the above effects between positive serum IgG and the monoclonal antibody group. Similarly, the monoclonal antibody could also increase the beating rates of cardiomyocytes, which was consistent with the previous report by Staudt *et al.* [14]. Furthermore, we demonstrated that the monoclonal antibody was able to induce the cardiomyocyte apoptosis dose-dependently. It also indicated that it is possible for autoantibody against β_1-adrenoceptor to induce myocardial injury through the apoptotic pathway.

Much evidence suggests that autoantibody against β_1-adrenoceptor displays the "agonist-like" activity without desensitization, which is very different from the β_1-adrenoceptor agonist, isoprenaline. It is known that excessive stimulation using isoprenaline leads to apoptosis [24]. Therefore, to further confirm that overstimulation of β_1-adrenoceptor by autoantibody might exactly induce myocardial apoptosis, we made another comparative study with isoprenaline. Our results showed that isoprenaline

significantly increased the beating rate of cultured cardiomyocytes and definitely induced cardiomyocyte apoptosis. According to previous research, the isoprenaline-induced apoptosis is mediated by activation of the cAMP/PKA pathways and voltage-dependent calcium influx, which induces cardiac hypertrophy and subsequent DCM [24]. Thus, it can be speculated that the signaling transduction pathway of autoantibody-induced apoptosis might be related to isoprenaline.

In conclusion, our results indicated that anti-β_1-adrenoceptor autoantibody could produce chronotropic effects and induce the apoptosis of cultured cardiomyocytes by overstimulation of β_1-adrenoceptors, which might be responsible for antibody-induced myocardial injury. All of these results provide promise for the development of potential clinical approaches in which Ig-adsorption therapy might substitute heart transplantation and become the standard therapy for DCM.

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