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β_1-Adrenoceptor antibodies induce apoptosis in adult isolated cardiomyocytes

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Abstract

β_1-Adrenoceptor autoantibodies are present in about 30% of patients suffering from dilated cardiomyopathy. The apoptotic effects mediated by β_1-adrenoceptor antibodies remain to be studied. Monoclonal antibodies were raised against a synthetic peptide corresponding to the second extracellular loop of the human β_1-adrenoceptor in *balb*/C mouse, and were characterized by enzyme immunoassay. Purified immunoglobulin G from nonimmunized animals (controls) did not influence the rate of apoptosis. β_1-Adrenoceptor antibodies caused a dose-related increase in apoptotic cells: annexin test (dilution 1:2: $21 \pm 1.1\%$ apoptotic cells vs. $4 \pm 0.4\%$ apoptotic cells in controls; $p < 0.01$); TdT-mediated dUTP nick end labeling (TUNEL) test (dilution 1:2: $26 \pm 2\%$ apoptotic cells vs. $10 \pm 2\%$ apoptotic cells in controls; $p < 0.01$). The effect of the β_1-adrenoceptor antibodies was blocked by the antigenic peptide and by the antagonist metoprolol (10 μmol/l). The apoptotic effect induced by isoproterenol was attenuated by the β_1-adrenoceptor antibody. After pre-incubation of cardiomyocytes with the protein kinase A inhibitor Rp-Adenosine-3′,5′-cyclic monophosphothioate triethylamine (RpcAMPS), β_1-adrenoceptor antibody was not capable of inducing an increase of the rate of apoptosis. β_1-Adrenoceptor antibodies induced apoptosis in adult rat cardiomyocytes via the protein kinase A cascade.
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1. Introduction

Circulating autoantibodies against the β_1-adrenoceptor are detected by enzyme-linked immunosorbent assay (ELISA) in approximately 30% of patients suffering from dilated cardiomyopathy (Magnusson et al., 1990; Fu et al., 1994). Analysis of β_1-adrenoceptor autoantibodies may provide markers for autoimmunological reactions occurring in dilated cardiomyopathy (Felix et al., 2000; Dörffel et al., 1997).

The pathological relevance of this autoantibody was assessed by in vivo investigation. One-year immunization of rabbits induced histopathological changes in the hearts of the immunized animals, with phenomena comparable to those found in dilated cardiomyopathy (Matsui et al., 1997). The incidence of the β_1-adrenoceptor autoantibody is higher in patients with poorer left ventricular ejection fraction (Jahns et al., 1999a,b). These findings argue for a pathogenic role of this autoantibody in dilated cardiomyopathy. The causal pathogenic mechanisms induced by this autoantibody, however, are unclear.

It has been demonstrated in both animal (Liu et al., 1995; Kubota et al., 1997) and human studies (Narula et al., 1996; Olivetti et al., 1997) that apoptosis occurs in heart failure. Apoptosis is a highly regulated cell deletion process characterized by nuclear and cellular fragmentations. It is known that apoptosis may play a pathophysiological role in heart failure, but the pathological relevance of the various factors that induce apoptosis (e.g., cytokines and catecholamines) remains unanswered (Narula et al., 2000; Kubota et al., 2001).

Various studies have indicated that catecholamines, acting via the β-adrenoceptor pathway, stimulate apoptosis in

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adult cardiomyocytes. This effect is mediated by activation of the protein kinase A (Communal et al., 1998; Singh et al., 2001).

Recent publications have shown that the β_1-adrenoceptor antibody acts as a partial agonist and induces its effects via the protein kinase A cascade (Staudt et al., 2001; Wallukat et al., 1995).

The apoptotic effects on cardiomyocytes of the antibodies against β_1-adrenoceptor, however, remain to be elucidated. The purpose of the present study, accordingly, was to investigate whether the antibodies raised against the second extracellular loop of the β_1-adrenoceptor induce apoptosis in adult isolated ventricular cardiomyocytes. In view of the heterogeneity of the human autoantibodies and their relative scarcity in sera, it was decided to raise monoclonal antibodies in mice using a peptide corresponding to the second extracellular loop of the β_1-adrenoceptor as immunogen.

2. Materials and methods

2.1. Peptides and production of monoclonal antibodies

Production of monoclonal antibodies took place as previously described (Mobini et al., 2000). A peptide (H26R, 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 sequences of the second extracellular loop of the human β_1-adrenoceptor was commercially synthesized by Vitrogen of Ontario, Canada. Purified immunoglobulin G (IgG) from nonimmunized animals was used for control purposes. The purified IgG concentration was determined by a protein assay kit (Pierce, IL, USA). The antibodies were subjected to pre-incubation dilution in medium 199 with L-glutamine (mod. Earl's salt, 0.2% bovine serum albumin (BSA), 5 mmol/l creatinine, 2 mmol/l L-carnitine, 5 mmol taurine, 25 mmol/l HEPES, pH = 7.4).

2.2. ELISA

ELISA was used to check the specificity of antibodies before their application (Mobini et al., 2000). Nunc (Roskilde, Denmark) microtitre plates were coated with solutions of 10 μg/ml peptide in 0.1 M Na_2CO_3/1% β-mercaptoethanol for 1 h at room temperature. After saturation of the wells with 3% Phosphor Milk Tween (PMT) buffer (3% skimmed milk/0.1% Tween 20 in phosphate-buffered saline, pH = 7.4), the monoclonal antibodies were added to the plates and incubated for 2 h at 37 °C. The antibodies were revealed by successive incubations for 1 h at 37 °C with biotinylated rabbit anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, San Diego, CA), and were diluted 1:1000 in 3% PMT and with a streptavidin-peroxidase conjugate (Jackson ImmunoResearch Laboratories) at 1:1000 dilution in the same buffer. After washing the wells in phosphate-buffered saline, H_2O_2-2,2′-azino-di-3-ethylbenzthiazoline sulphonate (Boehringer Mannheim, Germany), substrate was added and the absorbance was read at 405 nm in a Titertek (Flow, Irvine, UK) ELISA reader after 1 h.

2.3. Cell isolation

Adult rat cardiac myocytes were isolated as recently described (Kubin et al., 1999). Briefly, hearts were perfused for 3 min with oxygenated Krebs–Henseleit buffer (37 °C, pH = 7.4) containing the following (in mmol/l): 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 20 glucose, and 10 HEPES, at pH = 7.3. Typically, about 2×10^6 cells per rat heart were obtained, most of which (95%) showed the typical rod-shaped morphology with no blebs or granulations. The cells were then plated on four-well chamber-glass slides (Nunc, Naperville, IL, USA) that had been coated with 10 μg/ml laminin. Cells were then incubated at 37 °C for 24 h with antibodies in different dilutions.

2.4. Analysis of rate of apoptosis in cardiomyocytes

After incubation, apoptosis was detected by TdT-mediated dUTP nick end labeling (TUNEL) assay and the Annexin test.

2.4.1. TUNEL assay

DNA fragmentation is a typical sign of apoptosis and was detected in situ by the TUNEL test. Terminal deoxynucleotidyl transfer-mediated end labeling of fragmented nuclei was performed on cardiomyocytes in accordance with the manufacturer's protocol for cultured cells (Roche Diagnostics, Mannheim, Germany).

The cells were briefly washed with phosphate-buffered saline and fixed in 4% paraformaldehyde for 1 h at 15–25 °C. After washing with phosphate-buffered saline, the cells were incubated in permeabilizing solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The cells were then rinsed with phosphate-buffered saline and incubated with the TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. As a positive control, fixed and permeabilized cells were treated with DNAse I (1 mg/ml, Sigma) for 10 min to induce DNA strand breaks. Samples were directly observed under a fluorescence microscope. At least 10 fields were analyzed for apoptotic myocytes in each slide. The apoptotic index or percentage of apoptotic nuclei was calculated as (apoptotic nuclei/total nuclei) × 100%.

2.4.2. Annexin test

In the early stages of apoptosis, translocation of phosphatidylserine (PS) occurs, from the inner part of the plasma membrane to the outer layer—as a result of which,

PS becomes exposed at the external surface of the cell. Analysis of phosphatidylserine on the outer leaflet of apoptotic cell membrane is performed by using Annexin V-Fluos and propidium iodide for differentiation from necrotic cells. The Annexin V-Fluos assay (Roche Diagnostics) was performed according to the manufacturer's protocol. The cells were briefly washed in phosphate-buffered saline, followed by incubation of cells with Annexin V-Fluos in a HEPES buffer containing propidium iodide for cell surfaces. After 10 to 15 min, the apoptotic cells were also analyzed by fluorescence microscopy at 40× magnification. At least 10 fields were examined for apoptotic cells on each slide.

2.5. Statistical analysis of data

Results are expressed as mean values ± S.E.M. for n calculations. Effects of the dilutions of antibodies were analyzed using nonparametric repeated measures analysis of variance with data alignment. Post-hoc analyses were performed (Mann–Whitney U-tests) after overall testing.

3. Results

ELISA applied only in the plasma of immunized animals revealed antibodies against β_1-adrenoceptor with a titre over 1/10,000 (data not shown). Tests were performed to determine the influence of different dilutions on the rate of apoptosis for the monoclonal antibody against β_1-adrenoceptor in comparison with controls. Incubation of the rat cardiomyocytes with control antibodies ($n=6$) obtained from nonimmunized animals did not influence the rate of apoptosis. However, when the cells were incubated with antibodies against the second extracellular loop of β_1-adrenoceptor ($n=6$), the rate of apoptosis measured by Annexin testing demonstrated significant increase. The increase of apoptosis depended on dilution of the β_1-adrenoceptor antibody with experimental buffer (Fig. 1). When antibodies against the β_1-adrenoceptor were diluted to 1:2, apoptosis (measured by the Annexin test) increased by 21 ± 2.4% ($p<0.001$ vs. controls). These results were confirmed by the TUNEL test (Fig. 2)

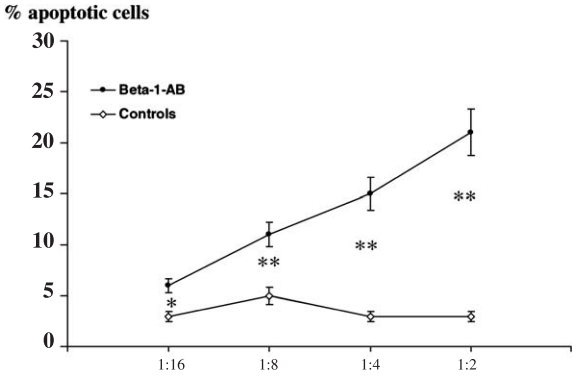

Fig. 1. Effects of various dilutions of antibodies (controls/β_1-adrenoceptor antibodies) on isolated rat cardiomyocytes. Changes of rate of apoptosis (measured by the Annexin test) after incubation of control antibodies (controls, $n=6$) and of β_1-adrenoceptor antibodies ($n=6$). The values (% apoptotic cells) are means ± S.E.M. for $n=6$ different myocytes. *$p<0.05$, **$p<0.01$ vs. controls.

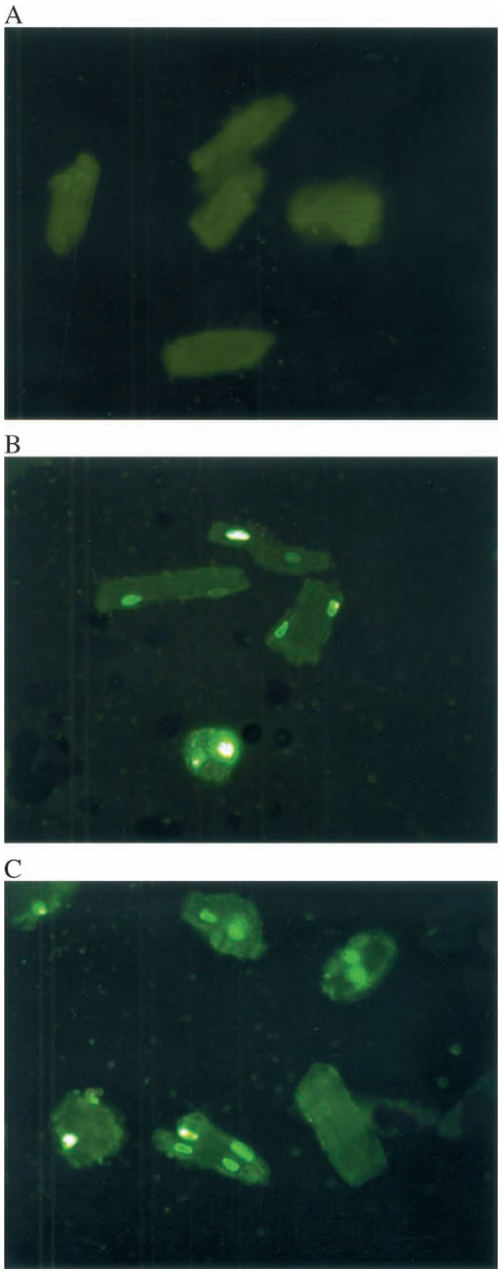

Fig. 2. Antibody against β_1-adrenoceptor increased frequency of DNA strand breaks as assessed by TUNEL staining. Adult rat cardiomyocytes were exposed to β_1-adrenoceptor antibodies (dilution 1:2) for 24 h and subjected to TUNEL staining as described in Materials and methods. (A) Control cells, (B) cells treated with DNase I (1 mg/ml), (C) cells treated with β_1-adrenoceptor antibodies.

(dilution 1:2; β_1-autoantibodies: 34 ± 1.3%/controls 12 ± 1.5% [$p < 0.001$ vs. controls]).

The effects of the β_1-adrenoceptor antibody (1:4) were significantly reduced in the presence of the β_1-adrenoceptor-selective antagonist metoprolol (10 μmol/l, $n = 6$) ($P < 0.01$ vs. β_1-adrenoceptor antibody 1:4) (Fig. 3). In order to block the specific antigenic binding sites, the β_1-adrenoceptor antibodies were incubated with a 30-fold surplus molar amount of the antigenic peptide of the second extracellular loop for 24 h at 4 °C. After this pre-incubation, the mixture was tested in a dilution of 1:4. The effect of the β_1-adrenoceptor antibodies was fully blocked after pre-incubation with the antigenic peptide ($P < 0.001$ vs. β_1-adrenoceptor antibody 1:4) (Fig. 3).

To test whether the antibodies induce their apoptotic effect via stimulation of the protein kinase A cascade, the protein kinase A inhibitor Rp-Adenosine-3′,5′-cyclic monophosphothioate triethylamine (RpcAMPS) was used to specifically block this pathway. After pre-incubation (20 min at room temperature) of cardiomyocytes with RpcAMPS (50 μmol/l, $n = 6$), the monoclonal β_1-adrenoceptor antibody (1:4) was not capable of inducing apoptosis of the cardiomyocytes ($P < 0.001$ vs. β_1-adrenoceptor antibody 1:4) (Fig. 3).

Metoprolol, RpcAMPS, and antigen did not influence the rate of apoptosis when the cardiomyocytes incubated with these substances separately. RpcAMPS and metoprolol furthermore eliminated the effects mediated by the agonist isoproterenol (10 μmol/l).

Additional experiments were performed to assess the interaction between the agonist isoproterenol and the β_1-adrenoceptor antibody. Antibodies against the β_1-adrenoceptor (dilution 1:4) induced apoptosis (15%) in rat cardiomyocytes. Isoproterenol (100 μmol/l) increased apoptosis to 24%. The isoproterenol-induced increase in apoptosis, however, was attenuated to 20% ($p < 0.01$ vs. isoproterenol alone) in the presence of the antibodies raised against the β_1-adrenoceptor (1:4) (Fig. 4).

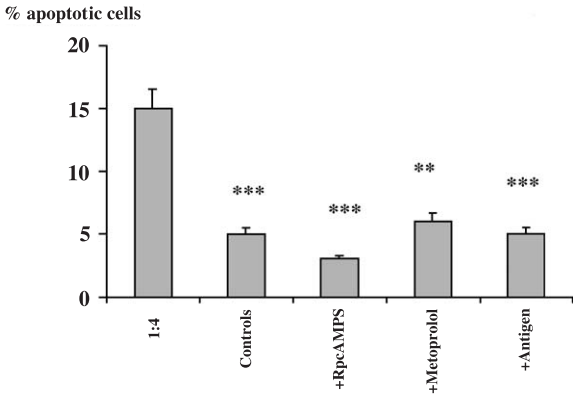

Fig. 3. Interaction of various substances (metoprolol 10 μmol/l, antigen, RpcAMPS 50 μmol/l) with β_1-adrenoceptor antibodies (1:4) on rat cardiomyocytes. Changes of rate of apoptosis (measured by the Annexin test) ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. β_1-adrenoceptor antibody.

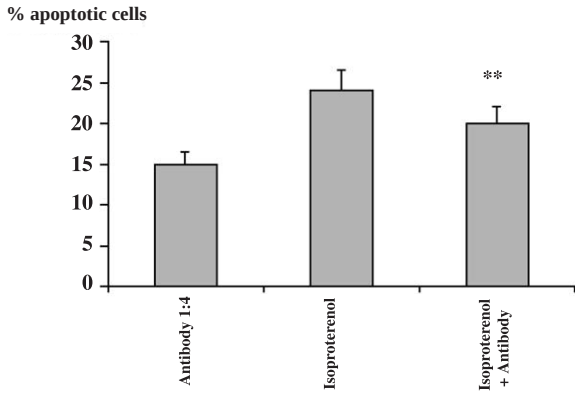

Fig. 4. Interaction between the agonist isoproterenol (100 μmol/l) and the β_1-adrenoceptor antibody (1:4). Isoproterenol (100 μmol/l) was tested separately ($n = 6$, left plot) and in the presence of the monoclonal β_1-adrenoceptor antibodies (1:4, $n = 6$, right plot). $^{**}P < 0.01$ vs. isoproterenol (100 μmol/l).

4. Discussion

Autoimmune responses to various myocardial antigens have been proposed as factors involved in the pathogenesis of patients suffering from dilated cardiomyopathy. A number of findings indicate that autoimmunological reactions against the β_1-adrenoceptor may also play a pathogenic role in patients with dilated cardiomyopathy (Limas et al., 1990). Autoimmune reactions against the β_1-adrenoceptor are not present in cardiomyopathies secondary to valvular or hypertensive heart disease: which argues for a pathogenetic role of this antibody in dilated cardiomyopathy (Jahns et al., 1999b). Furthermore, immunization of rabbits with a peptide corresponding to the second extracellular loop of the β_1-adrenoceptor induced histopathological changes in the hearts of the immunized animals, with phenomena comparable to those found in dilated cardiomyopathy (Matsui et al., 1997). Jahns et al. (1999a) have further demonstrated antibodies against various synthetic receptor peptides in 51% of patients suffering from dilated cardiomyopathy. Only the subgroup directed against the second extracellular loop likewise recognized native human β-adrenoceptors of the cell membrane. Antibodies from this subgroup demonstrated functional activity. In the present study, we analyzed the effects of monoclonal antibody raised against one specific epitope of the human β_1-adrenoceptor. About 31% of patients suffering from dilated cardiomyopathy develop antibodies against this epitope.

The apparently limited capacity for regeneration of myocytes in the adult heart suggests that continued myocyte loss due to apoptosis may contribute to progression of heart failure. Recent findings have suggested a critical role for apoptosis in a number of cardiovascular diseases (Haunstet-

ter and Izumo, 1998). The number of apoptotic myocytes increases in myocardium obtained from patients with end-stage heart failure and DCM (Olivetti et al., 1997). Experiments with cultured cardiac myocytes have demonstrated that apoptosis can be induced in vitro by several endogenous peptides that are augmented in the hypertrophied or failing myocardium: including tumour necrosis factor-alpha (Krown et al., 1996), angiotensin II (Kajstura et al., 1997), atrial natriuretic peptide (Wu et al., 1997), and catecholamines (Communal et al., 1998).

The present study discloses that antibodies against the second extracellular loop of the β_1-adrenoceptor induce apoptosis in adult isolated rat cardiomyocytes. This monoclonal antibody clearly demonstrated dose-dependent increases in apoptotic effects in cardiomyocytes. Inhibition of protein kinase A by RpcAMPS suppressed the apoptotic effect produced by the antibodies raised against the second extracellular loop of the β_1-adrenoceptor.

We recently demonstrated that monoclonal antibodies directed against the second loop of β_1-receptor are able to induce a dose-related increase of cAMP (Staudt et al., 2001). These data indicate that the adenylate cyclase/protein kinase system constitutes the pathway by which the antibody raised against the β_1-adrenoceptor displays its apoptotic effect.

β_1-Adrenoceptor antibodies attenuate the maximum apoptotic effect induced by the pure agonist isoproterenol. Antibodies against the β_1-adrenoceptor accordingly act as partial agonists. An increased level of catecholamines is found in patients with heart failure. Our experiments indicate that antibodies against the β_1-adrenoceptor can reduce the apoptotic effect of catecholamines.

β_1-Adrenoceptor antagonists can block the apoptotic effects of β_1-adrenoceptor antibodies. Inhibition of β_1-adrenoceptor autoantibodies may contribute to the beneficial effects of β-adrenoceptor blockade in chronic heart failure.

In conclusion, we have demonstrated that the β_1-adrenoceptor antibody induces a specific apoptotic effect via the protein kinase A cascade.

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