**β₁-Adrenergic Receptor Autoantibodies Mediate Dilated Cardiomyopathy by Agonistically Inducing Cardiomyocyte Apoptosis**

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**Background**—Antibodies to the β₁-adrenergic receptor (β₁AR) are detected in a substantial number of patients with idiopathic dilated cardiomyopathy (DCM). The mechanism whereby these autoantibodies exert their pathogenic effect is unknown. Here, we define a causal mechanism whereby β₁AR-specific autoantibodies mediate noninflammatory cardiomyocyte cell death during murine DCM.

**Methods and Results**—We used the β₁AR protein as an immunogen in SWXJ mice and generated a polyclonal battery of autoantibodies that showed selective binding to the β₁AR. After transfer into naive male hosts, β₁AR antibodies elicited fulminant DCM at high frequency. DCM was attenuated after immunoadsorption of β₁AR IgG before transfer and by selective pharmacological antagonism of host β₁AR but not β₂AR. We found that β₁AR autoantibodies shifted the β₁AR into the agonist-coupled high-affinity state and activated the canonical cAMP-dependent protein kinase A signaling pathway in cardiomyocytes. These events led to functional alterations in intracellular calcium handling and contractile function. Sustained agonism by β₁AR autoantibodies elicited caspase-3 activation, cardiomyocyte apoptosis, and DCM in vivo, and these processes were prevented by in vivo treatment with the pan-caspase inhibitor Z-VAD-FMK.

**Conclusions**—Our data show how β₁AR-specific autoantibodies elicit DCM by agonistically inducing cardiomyocyte apoptosis. (Circulation. 2007;116:399-410.)

**Key Words:** antibodies ■ apoptosis ■ autoimmunity ■ cardiomyopathy ■ heart failure ■ receptors, adrenergic, beta

Diopathic dilated cardiomyopathy (DCM) is a disease of putative autoimmune origin that frequently causes congestive heart failure. A sizable proportion of patients with DCM develop immune responses to cardiac self-antigens; thus, tissue-restricted proteins such as the β₁-adrenergic receptor (β₁AR) have been investigated as containing determinants for immune cell activation before or during idiopathic DCM.¹ ³ ⁷

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The β₁AR is a 7-transmembrane G-protein–coupled receptor abundantly expressed on cardiomyocytes (reviewed elsewhere¹⁰). Cognate catecholamine binding to the β₁AR transmits an intracellular signal through a canonical cAMP-dependent protein kinase A (PKA) pathway that drives functional cardiomyocyte contractile alterations as part of the sympathetic “fight-or-flight” response. Up to 33% of patients with DCM have been reported to produce detectable circulating autoantibodies directed against epitope regions of the β₁AR protein.¹¹ The pathogenic potential of β₁AR-specific autoantibodies was affirmed by recent studies in which recipient rodents developed DCM after passive transfer of β₁AR-specific antisera¹²,¹³ and by clinical trials showing hemodynamic benefit in patients with DCM after protein A immunoadsorption of serum.¹⁴,¹⁵

Although the qualitative effects of β₁AR autoantibodies on cardiomyocyte function and survival have been described in vitro,¹⁶–¹⁹ a causal mechanism defining how these β₁AR-specific antibodies exert their pathogenic effect in vivo is lacking. In this context, we investigated the pathogenesis of β₁AR autoantibody–induced heart disease using a newly developed murine model of DCM. We found that sera obtained from SWXJ mice immunized with the β₁AR protein are capable of inducing fulminant high-frequency DCM when transferred into naive SWXJ recipients. Development of DCM was dependent on selective binding of β₁AR-specific IgG to host β₁AR because immunoadsorption of β₁AR-specific antibodies prevented development of fulminant DCM. We found that sera elicited from β₁AR-specific autoantibodies against β₁AR protein before DCM

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specific IgG or treatment with a βAR but not a β2AR antagonist abrogated the development of DCM.

We found that βAR antibodies act as classic functional agonists, causing an increase in cardiomyocyte contractility (positive inotropy) and an acceleration in cardiomyocyte relaxation (positive lusitropy) mediated by changes in the handling of intracellular calcium (Ca2+). Sustained agonism of cardiomyocytes by βAR-specific autoantibodies elicits caspase-3 activation in vivo through the Ca2+- and cAMP-dependent PKA pathways. Taken together, our data define a novel mechanism whereby autoimmune responses directed against the βAR elicit DCM by inducing noninflammatory cardiomyocyte cell death.

Methods

Passive Transfer of DCM
Six- to 8-week-old SWXJ (H-2k) male mice (Jackson Laboratories, Bar Harbor, Maine) were immunized subcutaneously in the abdominal flank on day 0 with either 100 μg ovalbumin (OVA; Sigma, St Louis, Mo) or 100 μg human recombinant βAR (Perkin-Elmer, Boston, Mass) and 400 μg Mycobacterium tuberculosis H37RA (Difco, Detroit, Mich) in 200 μL of an emulsion of equal volumes of water and Freund’s adjuvant (Difco). Three weeks later, cell-free antisera were collected, pooled, and intravenously injected into naive recipient SWXJ male hosts at 200 μL per mouse. Recipient mice were euthanized at various times after transfer of antisera by CO2 asphyxiation followed by cervical dislocation.

For IgG depletion, antisera were passed through a PROSEP-G spin column (Millipore, Billerica, Mass) according to the manufacturer’s recommendations, and IgG depletion was confirmed by coating immunoassay plates (Nalge Nunc, Rochester, NY) with column eluate and performing a direct ELISA using goat anti-mouse IgG (Southern Biotechnology, Birmingham, Ala). For in vivo adenergic antagonism, mice were treated orally in the drinking water with 40 μg/d of the βAR antagonist CGP 20712 dichloride (Tocris, Ellisville, Mo) or the β2AR antagonist ICI 118551 (Tocris). All protocols were approved by the Cleveland Clinic Institutional Animal Care and Use Committee in compliance with the Public Health Service policy on humane care and use of laboratory animals.

Histological Analysis
Cardiac sections were stained with hematoxylin and eosin and read in a blinded manner as described previously. Criteria for the presence of DCM included increased ventricular chamber widths and increased ratios of heart weight to body weight, calculated by dividing the heart weight in milligrams by the body weight in grams. Ratios of nuclei staining positively for terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) fluorescein (Roche, Indianapolis, Ind) versus 4’6-diamidino-2-phenylindole dihydrochloride (DAPI) blue (Vector, Burlingame, Calif) were quantified in 6 high-powered fields per sample with ImagePro computer software (Media Cybernetics, Silver Spring, Md).

Echocardiography
Histological DCM was confirmed by functional measurements assessed by echocardiography. Mice were imaged in the lateral decubitus position. Echocardiography was performed with a Vivid 7 machine (GE Medical, Milwaukee, Wis). Gray-scale 2-dimensional and M-mode data were collected with a 14-MHz epicardial linear transducer and analyzed with Echopac personal computer analysis software (GE Medical).

ELISA and Western Blot Analysis
For direct ELISA, immunoassay plates (Nalge Nunc) were coated overnight at 4°C with 20 μg/mL βAR protein, and antisera diluted 1/1000 were overlaid at 4°C on coated plates. Serum antibody isotyping was performed according to the manufacturer’s instruc-

tions using the mouse MonoAB ID/SP ELISA kit (Zymed, South San Francisco, Calif). Serum brain natriuretic peptide (BNP) concentrations were measured with the BNP-32 Rat ELIA kit (Phoenix Pharmaceuticals, Belmont, Calif). Western blots of cardiac protein extracts were performed with a βAR-specific antibody (Abcam, Cambridge, UK), βAR antisera, or an antibody (1/1000) recognizing PKA-specific phosphorylated consensus sequence (RRXS, Abcam).21

βAR Competition Binding Experiments
Heart membrane proteins were prepared as previously described. Membrane protein concentrations were determined by the Bradford method. Competition binding experiments using the radioligand [125I]cyanopindolol (CYP; Amersham, Piscataway, NJ) were carried out in a final volume of 0.5 mL containing HEM buffer (20 mmol/L Hepes, pH 7.5, 1.4 mmol/L EGTA, and 12.5 mmol/L MgCl2, 150 mmol/L [125I] CYP (Perkin Elmer, Welle-

sley, Mass), cardiac cell membranes, and increasing concentrations of norepinephrine (0.1 mmol/L to 1 mmol/L, Sigma) in the presence or absence of βAR-specific antibody or OVA-specific control antibody at 1/20 000 dilutions. Antisera and membranes were preincubated for 1 hour at 25°C before the addition of the other components. The reactions were then incubated for another 60 minutes at 25°C, terminated by addition of 3 mL binding buffer at 4°C, and rapidly filtered and washed through Whatman GF/C glass fiber filters (Fisher Scientific, Pittsburgh, Pa) using a model 290 PHD cell harvester (Brandel, Gaithersburg, Md). No significant difference existed in total binding in the presence or absence of βAR antisera. Bound radioactivity was quantified with an autogamma 500 counter (Packard Instruments, Meriden, Conn). Binding data were analyzed with the iterative curve-fitting software program GraphPad Prism (GraphPad, San Diego, Calif). An F test using the sum of the squares determined whether the data fit statistically better to a 1- or 2-site model, and the percentages of high- and low-affinity sites were calculated.

Cardiomyocyte Cultures
Cardiomyocyte single-cell suspensions were obtained through collagenase perfusion as described and cultured in 24-well plates on glass coverslips precoated with 10 μg/mL laminin. Cardiomyocytes were suspended in serum-free Krebs-Henseleit buffer containing 118 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L MgCl2, 1.2 mmol/L KH2PO4, 1.2 mmol/L CaCl2, 37.5 mmol/L NaHCO3, and 16.5 mmol/L dextrose with a pH adjusted to 7.35. Cardiomyocytes were added 1 hour before the addition of antisera. Before use in vitro, complement components of βAR-specific and OVA-specific antisera were functionally depleted by 3 freeze-thaw cycles as confirmed by low cell surface detection of C5 on target cell membranes as previously described.

Pharmacological agonists and antagonists used in our experiments include 100 mmol/L CGP 20712 dichloride (Tocris), 100 mmol/L ICI 118551 (Tocris), 5 μmol/L PKI 14-22 inhibitor (EMD Biosciences, San Diego, Calif), 20 μmol/L H-89 (EMD Biosciences), 40 μmol/L ApoBlock (BD Biosciences, San Jose, Calif), 1 μmol/L nifedipine (Sigma), 1 μmol/L isoproterenol (Sigma), 10 μmol/L KN62 (Calbiochem, San Diego, Calif), and 0.5 μmol/L KN93 (Calbiochem).

Measurement of [Ca2+], Transients and Cardiomyocyte Shortening
Intracellular Ca2+ concentration [Ca2+], and cardiomyocyte shortening were simultaneously monitored as previously described. Briefly, ventricular cardiomyocytes at 5×104 cells/mL were incubated in Krebs-Henseleit buffer containing 2 μmol/L fura2/AM (Texas Fluorescence Labs, Austin, Tex). Fluorescence measurements were performed on single ventricular cardiomyocytes using a dual-wavelength spectrophotometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The cells also were illuminated with red light at a wavelength >600 nm for simultaneous measurement of cardiomyocyte shortening using a
video-edge detector (Crescent Electronics, Sandy, Utah). The video-edge detector was calibrated with a stage micrometer so that cell lengths during shortening and relengthening could be measured. Because calibration procedures rely on a number of assumptions, the ratio of the light intensities at the 2 wavelengths was used to measure qualitative changes in [Ca\(^{2+}\)]. Just before data acquisition, background fluorescence was measured and automatically subtracted from the subsequent experimental measurement. The fluorescence sampling frequency was 100 Hz, and data were collected using a software package from Photon Technology International (Lawrenceville, NJ). Fluorescence data for the [Ca\(^{2+}\)], transients and cardiomycocyte shortening were imported into Laboratory View (National Instruments, Austin, Tex), and both the [Ca\(^{2+}\)], transients and cardiomycocyte contractile responses were analyzed synchronously and simultaneously.

For analysis of [Ca\(^{2+}\)] and shortening data, the following variables were calculated for each individual contraction: resting [Ca\(^{2+}\)], and cell length; peak [Ca\(^{2+}\)], and cell length; change in [Ca\(^{2+}\)], (peak [Ca\(^{2+}\)], minus resting [Ca\(^{2+}\)],) and twitch amplitude; time to peak (T\(_{\text{p}}\)) for [Ca\(^{2+}\)], and shortening and time to 50% or 90% (T\(_{0.5\text{relax}}\), T\(_{0.9\text{relax}}\)) resting [Ca\(^{2+}\)], and relengthening. Variables from 10 contractions were averaged to obtain mean values at baseline and in response to the various interventions. Averaging the variables over time minimized beat-to-beat variation.

Quantification of dead and apoptotic cells was determined in a blinded manner on the basis of manual counts of Trypan blue exclusion and fluorescent staining, respectively, of 3 to 5 separate high-power fields per sample containing >200 cells per field. Apoptosis was determined by fluorescence TUNEL staining (Roche) and by Annexin V PE staining (BD Biosciences) according to the manufacturers’ specifications with a modified 2-hour permeabilization period.

**Caspase-3 and PKA Enzyme Activity**

Caspase-3 activity was measured with the ApoAlert Caspase-3 Assay Plate (Clontech, Mountain View, Calif) according to the manufacturer’s instructions with a modified 60-minute reaction time at 37°C. PKA activity was measured with the PepTag Non-Radioactive cAMP Dependent Protein Kinase Assay (Promega, Madison, Wis) and calculated per the manufacturer’s specifications. Spectrophotometric measurements at 570 nm were performed on 40 μg/mL cardiac tissue after a 30-minute reaction time at room temperature.

**Flow Cytometry Competition Assay**

Cardiomyocyte single-cell suspensions were harvested from 24-well plates via mechanical disruption with a pipette and incubated with varying dilutions of human recombinant β\(_{1}\)AR or β\(_{2}\)AR protein for 20 minutes before staining with β\(_{1}\)AR antisera at 1/50 dilution and cardiac myosin heavy-chain antibody (Abcam) at 1/100 dilution followed, respectively, by PE-conjugated goat anti-mouse IgG (BD Biosciences) and FITC conjugated goat anti-rabbit IgG (Vector). Results were analyzed with a BD FACSCalibur flow cytometer (BD Biosciences).

**Biostatistical Analysis**

The nonpaired Student t test was used to compare differences in frequencies of apoptotic nuclei, caspase-3, and PKA enzyme activity; ratios of heart weight to body weight; echocardiography functional data; high-affinity receptor states; peak [Ca\(^{2+}\)], transients; peak cardiomycocyte shortening; and BNP serum levels in mice or cultures treated with β\(_{1}\)AR versus OVA antisera.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Generation of β\(_{1}\)AR-Specific Autoantibodies**

To generate β\(_{1}\)AR autoantibody, we immunized male SWXJ mice with recombinant human β\(_{1}\)AR protein. At 2, 4, and 8 weeks after immunization, the presence of serum IgG binding to β\(_{1}\)AR protein at a serum dilution of 1/1000 was determined by direct ELISA. Immunization with the β\(_{1}\)AR protein caused a progressive increase over time in antigen-specific IgG (Figure 1A). We found that β\(_{1}\)AR-specific autoantibodies consisted predominantly of IgG2a and IgG2b isotypes with relatively low levels of the IgG1 isotype, consistent with a Th1-like antibody phenotype\(^{26}\) (Figure 1B).

We next determined the specificity of autoantibody binding to the β\(_{1}\)AR by Western blot analysis. We found that β\(_{1}\)AR specific antisera at a 1/1000 dilution and a commercially purchased positive control β\(_{1}\)AR-specific antibody showed similar Western blot staining patterns against cardiac protein extracts (Figure 1C, lanes 1 and 2, respectively). No signal was detected when β\(_{1}\)AR antisera were blotted against β\(_{2}\)AR recombinant protein (Figure 1C, lane 3). Specific immunostaining of endogenous β\(_{1}\)AR by β\(_{1}\)AR antisera was attenuated with increasing concentrations of soluble recombinant β\(_{1}\)AR (Figure 1C, top right blot) but not β\(_{2}\)AR (Figure 1C, bottom right blot) added as competition.

As further confirmation of the specificity of the β\(_{1}\)AR antisera, cardiomyocytes were double stained with β\(_{1}\)AR antisera and cardiac myosin heavy-chain antibody in the presence of varying dilutions of competing β\(_{1}\)AR or β\(_{2}\)AR protein. We observed diminishing frequencies of the double-staining β\(_{1}\)AR-positive and cardiac myosin–positive cells with increasing concentration of added β\(_{1}\)AR (Figure 1D, top row) but not β\(_{2}\)AR protein (Figure 1D, bottom row). From these studies, we concluded that antisera generated against the β\(_{1}\)AR protein contained substantial levels of Th1-like skewed autoantibodies that showed a pattern of β\(_{1}\)AR-specific binding virtually identical to commercially available β\(_{1}\)AR-specific antibodies.

**β\(_{1}\)AR-Specific Autoantibodies Cause DCM**

To determine the pathogenic potential of β\(_{1}\)AR autoantibodies, immune antisera were injected intravenously into naive male SWXJ hosts. At 3 weeks after passive transfer, we found that 7 of 12 mice receiving β\(_{1}\)AR-specific antisera (Figure 2A, top) developed gross cardiac enlargement and biventricular dilatation consistent with DCM. None of 8 mice receiving control OVA-specific antisera developed DCM (Figure 2A, bottom). Evolution of the DCM phenotype in affected mice occurred without any observed increase in cardiac mononuclear cell infiltration. DCM occurred in mice (3 of 4) injected with the IgG-enriched fraction of β\(_{1}\)AR antisera (Figure 2B, top) but was substantially abrogated in mice (1 of 8) injected with β\(_{1}\)AR antisera depleted of IgG by immunoadsorption (Figure 2B, bottom). In addition, DCM occurred in mice (4 of 8) receiving 40 μg/d ICI 118551, a selective β\(_{2}\)AR antagonist (Figure 2C, top), but was substantially abrogated in mice (1 of 8) receiving 40 μg/d CGP 20712, a selective β\(_{1}\)AR antagonist (Figure 2C, bottom).

Correlate echocardiographic measurements were taken at multiple time points after transfer of β\(_{1}\)AR antisera. Mice injected with β\(_{1}\)AR- but not OVA-specific antisera showed functional changes consistent with DCM, with significantly reduced fractional shortening (Figure 2D, left) and ejection fractions of mice receiving β\(_{1}\)AR antisera. Cardiomyocytes from these mice showed a significant increase in apoptotic nuclei stained with fluorescein TUNEL (Figure 2D, right).
fractions (Figure 2D, middle) on days 17 and 28 ($P < 0.02$) and significantly increased left ventricular end-systolic diameter (Figure 2D, right) on day 28 ($P < 0.03$).

Cardiomegaly was assessed by measuring the ratios of heart weight (mg) to body weight (g) in all treatment groups. Our measurements showed significant differences in cardiomegaly between $\beta_1$AR antisera– and OVA antisera–treated mice ($P < 0.001$), between IgG fraction– and IgG-depleted fraction–treated mice ($P = 0.04$), and between $\beta_1$AR antisera–injected mice treated with the $\beta_1$AR antagonist ICI 118551 and the $\beta_1$AR antagonist CGP 20712 ($P = 0.03$; the Table). Together, our data indicate that $\beta_1$AR IgG autoantibodies are sufficient to mediate DCM in vivo and that the development of DCM is contingent on specific binding of autoantibody to endogenous cardiac $\beta_1$AR.

$\beta_1$AR-Specific Autoantibodies Act Agonistically

We hypothesized that $\beta_1$AR autoantibodies may be exerting a direct toxic effect on host cardiomyocytes, possibly through alteration of cardiomyocyte signaling. To examine this possibility, cardiac surface membranes with abundant $\beta_1$AR were isolated and exposed to $\beta_1$AR antisera in the presence of the radioactive $\beta_1$AR antagonist, CYP, and competing dilutions of norepinephrine. A sigmoid-shaped binding curve was constructed from 5 of these competition assays, and the percentage of receptors in the high-affinity state was calculated (Figure 3A, left). We found that $\beta_1$AR antisera caused a leftward shift of the competition binding curve consistent with an increase in the proportion of receptors in the high-affinity state. Computer modeling indicated that the $\beta_1$AR antisera caused a statistically significant increase in the proportion of $\beta_1$AR receptors in the high-affinity state.
in the frequency of high-affinity β₁AR conformations (Figure 3A, right). The observed high-affinity conformational shift implied that endogenous β₁AR had become coupled to G protein, thereby increasing intracellular second messengers. To determine whether the intracellular signal derived from β₁AR antibody binding is capable of altering cardiomyocyte contractile function, untreated male SWXJ cardiomyocytes were freshly isolated and loaded with fura2/AM for measurement of \([\text{Ca}^{2+}]_i\). Simultaneous measurements of \([\text{Ca}^{2+}]_i\) (Δ340/380 ratio) and cardiomyocyte contractility (video-edge detection) were recorded in individual cells paced at 0.5 Hz before and after exposure to β₁AR-specific antisera (1/1000), OVA-specific antisera (1/1000), or isoproterenol (1 μmol/L). Exposure of cardiomyocytes to β₁AR antisera (Figure 3B, right top and bottom) but not OVA antisera (Figure 3B, left top and bottom) increased peak \([\text{Ca}^{2+}]_i\) and peak shortening comparable to isoproterenol-treated cardiomyocytes (Figure 3B, middle top and bottom). An overlay depicting an average of 10 individual \([\text{Ca}^{2+}]_i\) transients (Figure 3C, top left) and contractile events (Figure 3C, bottom left) clearly demonstrates the positive inotropic and lusitropic effects of β₁AR antisera on cardiomyocyte function. Compared with OVA antisera–treated controls, exposure of cardiomyocytes to...
$\beta_1$AR antisera resulted in an increase in cardiomyocyte peak $[Ca^{2+}]$, $\Delta^{340/380}$ ratio of 0.5 for OVA antisera versus 0.7 for $\beta_2$AR antisera) and an increase in twitch amplitude (2.50 $\mu$m for OVA antisera versus 6.4 $\mu$m for $\beta_2$AR antisera).

Compared with treatment of cardiomyocytes with OVA antisera, substantially increased $[Ca^{2+}]$ (Figure 4A, top left, and Figure 4B), prolonged exposure to agonists. Compared with cultures treated with OVA antisera for 1 hour with the pan-caspase inhibitor ApoBlock (Figure 4B), suggesting that apoptosis was the predominant mode of cell death.

### $\beta_1$AR-Specific Autoantibodies Induce Cardiomyocyte Apoptosis In Vitro

Sustained agonism induces cell death in numerous cell types. Additionally, persistent isoproterenol-induced agonism of $\beta_1$AR/$\beta_2$AR leads to cardiomyocyte cell death. To determine whether $\beta_1$AR autoantibodies affect cardiomyocyte survival, single-cell suspensions of cardiomyocytes were cultured with varying dilutions of complement-depleted $\beta_1$AR-specific antisera and pharmacological inhibitors. After 24 hours, cardiomyocytes were stained with a 1/50 dilution of Trypan blue, and the cumulative percentage of cell death was measured by Trypan blue exclusion in 3 to 5 high-power fields. Compared with cultures treated with OVA antisera (Figure 4A, top left, and Figure 4B), prolonged exposure to $\beta_2$AR-specific antisera caused a dose-dependent increase in cell death (Figure 4A, right column, and Figure 4B) similar to that seen in cultures treated with the nonselective $\beta_1$AR/$\beta_2$AR agonist isoproterenol (Figure 4B). Cumulative cell death induced by $\beta_2$AR antisera (1/200) was attenuated by pretreatment with 100 nmol/L CGP 20712, a selective $\beta_1$AR antagonist, but not with 100 nmol/L ICI 118551, a selective $\beta_2$AR antagonist (Figure 4B). Most notably, total cell death in response to $\beta_2$AR antisera was prevented by pretreatment of cardiomyocytes for 1 hour with the pan-caspase inhibitor ApoBlock (Figure 4B), suggesting that apoptosis was the predominant mode of cell death.

### Table: Ratios of Heart Weight to Body Weight

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HW/BW</th>
<th>$P$</th>
</tr>
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<tbody>
<tr>
<td>$\beta_1$AR antisera alone (n=12)</td>
<td>5.3±0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>OVA antisera alone (n=8)</td>
<td>3.9±0.2</td>
<td>-</td>
</tr>
<tr>
<td>IgG fraction of $\beta_1$AR antisera  (n=4)</td>
<td>4.9±0.5</td>
<td>0.04</td>
</tr>
<tr>
<td>IgG-depleted $\beta_2$AR antisera  (n=8)</td>
<td>4.0±0.2</td>
<td>-</td>
</tr>
<tr>
<td>$\beta_1$AR antisera + $\beta_2$-antagonism  (n=8)</td>
<td>5.0±0.6</td>
<td>0.03</td>
</tr>
<tr>
<td>$\beta_1$AR antisera + $\beta_2$-antagonism  (n=8)</td>
<td>3.9±0.3</td>
<td>-</td>
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</table>

Heart weight (HW) was measured in milligrams; body weight (BW) was measured in grams. Values are mean±SE.
To directly assess apoptotic frequencies, cardiomyocytes were examined for TUNEL and Annexin V staining. Compared with a 1/200 dilution of OVA antisera–treated cultures (Figure 4C, top left, and Figure 4D), cardiomyocytes treated with a 1/200 dilution of β₁AR antisera showed a higher frequency of apoptosis as shown by TUNEL staining (Figure 4C, bottom left, and Figure 4D). Frequencies of apoptotic cells were substantially decreased by addition of the β₁AR antagonist CGP 20712 or the pan-caspase inhibitor ApoBlock (TUNEL, bottom right) but not by addition of the β₂AR antagonist ICI 118551 (Annexin V, middle right). D, TUNEL and Annexin V results show that pan-caspase or β₁AR antagonism but not β₂AR antagonism protects cardiomyocytes from β₁AR antisera–induced apoptosis. All experiments shown are representative of at least 4 repetitions. All images were taken originally at ×200 magnification. All error bars show mean ± SE.

Figure 4. β-AR-specific autoantibodies induce cardiomyocyte apoptosis in vitro. Cardiomyocyte cultures were treated with pharmacological inhibitors before treatment with varying dilutions of OVA- or β-AR-specific antisera. A, After 24 hours, viability of cardiomyocytes as determined by Trypan blue exclusion was decreased in cultures treated with varying dilutions of β₁AR-specific antisera vs cultures treated with OVA antisera. B, Total cell death induced by a 1/200 dilution of β₁AR-specific antisera was similar to cultures treated with the β₁AR/β₂AR agonist isoproterenol (ISO; 1 μmol/L), was unaffected by pretreatment with the β₂AR antagonist ICI 118551, but was substantially attenuated by pretreatment with the β₁AR antagonist CGP 20712 or the pan-caspase inhibitor ApoBlock. C, Specific staining for apoptosis of cardiomyocyte cultures showed that compared with 1/200 OVA antisera–treated controls (TUNEL, top left), cardiomyocytes treated with a 1/200 dilution of β₁AR antisera showed a higher frequency of apoptosis (TUNEL, bottom left) that was substantially decreased by addition of the β₁AR antagonist CGP 20712 (Annexin V, top right) or the pan-caspase inhibitor ApoBlock (TUNEL, bottom right) but not by addition of the β₂AR antagonist ICI 118551 (Annexin V, middle right).
antagonist CGP 20712 (Figure 4C, top right, and Figure 4D) but not by addition of the β2AR antagonist ICI 118551 as shown by Annexin V staining (Figure 4C, middle right, and Figure 4D). β2AR antisera–induced apoptosis as measured by TUNEL staining was prevented by pretreatment of cardiomyocytes for 1 hour with the pan-caspase inhibitor ApoBlock (Figure 4C, bottom right, and Figure 4D). Taken together, our data indicate that sustained agonism by β2AR autoantibodies caused cardiomyocyte apoptosis.

β1AR-Specific Autoantibodies Induce Cardiomyocyte Apoptosis In Vivo

We next determined whether apoptosis of endogenous cardiomyocytes occurs in vivo in mice treated with β1AR antisera. One week after transfer of β1AR- or OVA-specific antisera into naive SWXJ male mice, hearts were examined by TUNEL staining for the presence of apoptotic nuclei. Compared with hearts from OVA antisera–treated mice, hearts from β1AR antisera–treated mice showed an increased frequency of apoptotic TUNEL-positive green nuclei versus nonapoptotic DAPI-positive blue nuclei (Figure 5A through 5C). Digital quantification of TUNEL-stained green nuclei showed significantly increased (P<0.01) frequencies of apoptotic cardiomyocytes on days 1, 3, and 7 after transfer of antisera in hearts from mice receiving β1AR antisera vs OVA antisera. For all time points, n=3. E, Hearts taken at several time points after injection of mice with β1AR antisera showed significantly increased caspase-3 enzymatic activity (P<0.01) vs hearts from mice treated with OVA antisera. For all time points, n=3. F, DCM incidence was substantially decreased in mice treated with the pan-caspase inhibitor Z-VAD-FMK. The decreased incidence of DCM in Z-VAD-FMK–treated mice vs vehicle-treated mice was accompanied by significantly decreased frequencies of apoptotic nuclei (P<0.0001) at day 1 after transfer (top right), significantly decreased (P<0.01) ratios of heart weight to body weight (middle right), and significantly decreased (P<0.01) serum BNP levels (bottom right). For all data points, n=6. All error bars show mean±SE.
Concurrently, we also determined levels of caspase-3 activity in recipient hearts at various times after transfer of βAR- and OVA-specific antisera and found that hearts from mice treated with βAR antisera showed significantly increased caspase-3 enzymatic activity (P<0.001) compared with hearts from mice treated with OVA antisera (Figure 5E). The caspase-3 activity in hearts from mice treated with βAR antisera was significantly increased at the 12-hour, 24-hour, 4-day, and 7-day time points after transfer of antisera (P<0.01).

To determine the significance of apoptosis in the development of DCM, male hosts were treated with the pan-caspase inhibitor Z-VAD-FMK or a vehicle control solution after transfer of βAR antisera. At 3 weeks, recipient hearts and sera were analyzed. Mice treated with vehicle developed a high incidence (4 of 6) of DCM (Figure 5F, top left), whereas DCM incidence was substantially decreased (1 of 6) in mice treated with Z-VAD-FMK (Figure 5F, bottom left). The decreased incidence of DCM in Z-VAD-FMK–treated mice was accompanied by a significant reduction (P<0.0001) in the number of TUNEL-positive apoptotic nuclei 1 day after transfer of βAR antisera (Figure 5F, top right), by a significantly reduced ratio of heart weight to body weight (P<0.01; Figure 5F, middle right), and by significantly decreased serum levels of BNP, a marker for heart failure.12 (P<0.01; Figure 5E, bottom right). Taken together, our data indicate that βAR autoantibodies induce cardiomyocyte apoptosis in vivo and that apoptosis is required for βAR autoantibody–mediated DCM.

**βAR Autoantibody–Induced Apoptosis and DCM Are PKA Dependent**

A recent study has shown that agonism of βAR with the nonselective βAR/βAR agonist isoproterenol induces cardiomyocyte apoptosis by a Ca
\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII)–associated pathway independently of canonical cAMP-dependent PKA signaling.30 Therefore, we investigated whether the cAMP-dependent PKA pathway was responsible for providing the proapoptotic signal in βAR antisera–treated cardiomyocytes.

Single-cell suspensions of male SWXJ cardiomyocytes were pretreated for 1 hour with 40 μmol/L H-89, a nonselective inhibitor of βAR/βAR pathways. Twenty-four hours later, cardiomyocyte cell death as measured by Trypan blue exclusion and apoptosis as measured by TUNEL and Annexin V immunostaining were reduced to levels observed in untreated control cultures (Figure 6A), indicating that H-89 completely rescued cardiomyocytes from βAR antisera–induced apoptosis (P<0.001).

To determine whether the cAMP-dependent PKA pathway was responsible for mediating this apoptotic rescue, selective inhibitors of either the PKA/cAMP-dependent or CaMKII-dependent pathway were added to cardiomyocyte cultures before the addition of βAR antisera. A PKA-specific inhibitor, PKI 14-22 (5 μmol/L), and an inhibitory cAMP analogue, RP-8-CPT-cAMPS (100 μmol/L), were added to inhibit the PKA/cAMP-dependent pathway. The selective CaMKII inhibitors KN-62 (10 μmol/L) and KN-93 (0.5 μmol/L) were added to inhibit the CaMKII-dependent pathway. At 20 hours, we found that specific inhibition of cAMP-dependent PKA signaling completely rescued cardiomyocytes from βAR antisera–induced apoptotic cell death as measured by frequencies of TUNEL– or Annexin V–positive cells (P<0.001; Figure 6A). However, selective CaMKII inhibition resulted in only a mild nonsignificant rescue from cell death and apoptosis, indicating that βAR autoantibodies induce cardiomyocyte apoptosis predominantly by activating the canonical cAMP-dependent PKA pathway.

Activation of the cAMP-dependent PKA pathway results in phosphorylation of multiple protein substrates that functionally increase [Ca
\(^{2+}\)].8–10 Because we observed an increase
in peak $[\text{Ca}^{2+}]$, after exposure to $\beta_1\text{AR}$ antisera (Figure 3B) and because disruption in intracellular $\text{Ca}^{2+}$ homeostasis elicits apoptosis, we hypothesized that inhibiting peak $[\text{Ca}^{2+}]$ would similarly prevent cardiomyocyte cell death. To test this hypothesis, cardiomyocytes were pretreated with the L-type $\text{Ca}^{2+}$ channel antagonist nifedipine 1 hour before the addition of $\beta_1\text{AR}$ antisera. At 24 hours, we found that nifedipine inhibition of $\text{Ca}^{2+}$ entry into cardiomyocytes abrogated total cell death and apoptosis to levels similar to those observed in PKI 14-22– and RP-8-CPT-cAMPS–pretreated cultures (Figure 6A). A similar level of rescue from apoptotic cell death was observed when $\text{Ca}^{2+}$ was buffered in the media with 5 mmol/L EGTA before the addition of $\beta_1\text{AR}$ antisera (data not shown). Taken together, our results indicate that $\beta_1\text{AR}$ antisera–induced cardiomyocyte apoptotic cell death is mediated through the cAMP-dependent PKA pathway, likely through alterations in $\text{Ca}^{2+}$ homeostasis.

We next determined whether PKA activation occurred in vivo during the development of $\beta_1\text{AR}$ antisera–induced DCM. To this end, cardiac proteins were extracted for analysis of PKA enzyme activity at various time points after injection of naive recipient mice with either $\beta_1\text{AR}$-specific or OVA-specific antisera. We found that PKA-specific enzyme activity was increased in hearts from mice treated with $\beta_1\text{AR}$ antisera at all the time points examined compared with hearts from OVA antisera–treated mice (Figure 6B). Moreover, Western blot analysis showed that hearts from mice treated with $\beta_1\text{AR}$ antisera had increased levels of phosphorylated PKA substrates at 1-, 2-, and 3-hour time points after injection with antisera (Figure 6C). The increase in phosphorylated substrates was dependent on $\beta_1\text{AR}$-specific binding because levels of protein phosphorylation decreased with concurrent $\beta_1\text{AR}$-selective pharmacological blockade with the $\beta_1\text{AR}$ antagonist CGP 20712 (data not shown). Taken together, our data indicate that $\beta_1\text{AR}$ autoantibodies initiate intracellular cAMP-dependent PKA signaling in cardiomyocytes in vivo.

**Discussion**

In the present study, we generated a polyclonal pool of $\beta_1\text{AR}$-specific antibodies that elicited high-frequency DCM in vivo through endogenous agonist-induced apoptosis of native cardiomyocytes. Our studies demonstrate the central role of apoptosis in the development of $\beta_1\text{AR}$ antibody–induced DCM and reveal a mechanism whereby autoimmune responses to a cardiac self-antigen, the $\beta_1\text{AR}$ protein, incite end-organ cell death through a noninflammatory mechanism.

Autoimmune responses directed against cardiac receptor signaling elements or contractile apparatus proteins play a significant role in the pathogenesis of DCM. Our studies have demonstrated a definitive pathogenic mechanism of $\beta_1\text{AR}$-specific antibodies in vivo that was lacking in prior studies using cardiomyocyte cultures. On the basis of this work, idiopathic DCM can be considered in the same light as other autoimmune diseases such as myasthenia gravis and Grave’s disease in which autoantibody recognition of protein receptors pathologically modulates parenchymal organ function. Indeed, autoantibodies directed against a multiplicity of cardiac antigens, including cardiac myosin, the $m_2$-muscarinic acetylcholine receptor, cardiac laminin, and the F1 ATP synthase, have been documented in patients with idiopathic DCM, and because these target autoantigens physiologically modulate cardiac function, antibody self-association to 1 or more of these proteins may represent a significant mechanism toward the overall pathogenesis of idiopathic DCM.

Most notably, we observed that autoantibody-induced cardiomyocyte apoptosis and pathological dilatory remodeling were completely reversed by in vivo inhibition of caspase activity with Z-VAD-FMK. This observation highlights the critical role of apoptosis in the pathogenesis of $\beta_1\text{AR}$ antibody–induced DCM and suggests that antibody-mediated proinflammatory mechanisms such as antibody-dependent cell-mediated cytolysis or complement-dependent cytolysis likely play relatively minor roles in the development of disease. In this regard, agonist-induced cardiomyocyte apoptosis may represent one of several noninflammatory modes of antibody-mediated pathology in DCM. Natural antibodies, composed of an evolutionarily conserved repertoire of broadly self-reactive IgM, for example, may promote tissue destruction through noninflammatory means, including direct target antigen proteolysis and target antigen opsonization. Thus, inflammatory destruction of cardiomyocytes may not necessarily constitute an obligate pathogenic component of DCM in which, especially in chronic disease, antibody-mediated end-organ destruction may persist long after immune self-tolerance has been established and cellular inflammatory processes have been attenuated.

Although species-defined differences almost always exist in immune recognition of identical proteins, it is important to note that our SWXJ mice developed autoimmune heart failure in response to human recombinant $\beta_1\text{AR}$. Thus, in our study, mouse antibody recognition of human $\beta_1\text{AR}$ epitopes resulted in DCM, implying that identification of such epitopes may likely have relevant implications for human disease mediated by antibodies to the $\beta_1\text{AR}$. Within the polyclonal pool of $\beta_1\text{AR}$ autoantibodies used in this study, a high degree of antibody heterogeneity is likely with regard to antigen binding sites on the $\beta_1\text{AR}$ protein, analogous to the main immunogenic region on the nicotinic acetylcholine receptor in myasthenia gravis. Because antibodies are structurally large molecules relative to native $\beta_1\text{AR}$ catecholamine ligands, it is likely that binding sites for $\beta_1\text{AR}$-specific autoantibodies are confined to portions of the extracellular loop domains of the $\beta_1\text{AR}$ protein. These loop domains have previously been shown to initiate G-protein–coupled signaling and represent putative autoantibody binding sites, in addition to the canonical catecholamine signaling domain buried within the 7-transmembrane pore of the $\beta_1\text{AR}$ protein, which is likely inaccessible to autoantibody binding because of molecular size constraints.
The low frequency of apoptotic nuclei observed in our study after βAR antisera transfer is in close alignment with a previous study showing that apoptosis in as few as ≈230 per 1×10^6 cardiomyocytes is sufficient to cause DCM with complete penetrance. Previous studies in humans with heart failure indicated much higher frequencies of apoptotic nuclei. However, given the lack of functional redundancy of the heart as an organ and with limited regenerative capacity of its constituent cardiomyocytes, it is unlikely that the heart can functionally support high frequencies of apoptotic events at any given point in time, especially during the often chronic course of heart failure. Our data instead support the notion that chronic, low-level cardiomyocyte apoptosis incites DCM and heart failure.

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Disclosures

None.

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