Cardiomyocyte apoptosis in autoimmune cardiomyopathy: mediated via endoplasmic reticulum stress and exaggerated by norepinephrine

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Mao W, Fukuoka S, Iwai C, Liu J, Sharma VK, Sheu S-S, Fu M, Liang C-S. Cardiomyocyte apoptosis in autoimmune cardiomyopathy: mediated via endoplasmic reticulum stress and exaggerated by norepinephrine. Am J Physiol Heart Circ Physiol 293: H1636–H1645, 2007. First published June 1, 2007; doi:10.1152/ajpheart.01377.2006.—Evidence suggests that the autoimmune cardiomyopathy produced by a peptide corresponding to the sequence of the second extracellular loop of the β1-adrenergic receptor (β1-ECII) is mediated via a biologically active anti-β1-ECII antibody, but the mechanism linking the antibody to myocyte apoptosis and cardiac dysfunction has not been well elucidated. Since the β1-ECII autoantibody is a partial β1-agonist, we speculate that the cardiomyopathy is produced by the β1-receptor-mediated stimulation of the CaMKII-p38 MAPK-ATF6 signaling pathway and endoplasmic reticulum (ER) stress, and that excess norepinephrine (NE) exaggerates the cardiomyopathy. Rabbits were randomized to receive β1-ECII immunization, sham immunization, NE pellet, or β1-ECII immunization plus NE pellet for 6 mo. Heart function was measured by echocardiography and catheterization. Myocyte apoptosis was determined by terminal deoxy transferase-mediated dUTP nick-end labeling and caspase-3 activity, whereas CaMKII, MAPK family (JNK, p38, ERK), and ER stress signals (ATF6, GRP78, CHOP, caspase-12) were measured by Western blot. Increased CaMKII activity, increased phosphorylation of p38 MAPK, and GRP78 and CHOP and increased cleavage of caspase-12, as well as increased CaMKII activity, increased phosphorylation of p38 MAPK, and nucleus translocation of cleaved ATF6. NE pellet produced additive effects. In addition, KN-93 and SB 203580 abolished the induction of ER stress and cell apoptosis produced by the β1-ECII antibody in cultured neonatal cardiomyocytes. Thus ER stress occurs in autoimmune cardiomyopathy induced by β1-ECII peptide, and this is enhanced by increased NE and caused by activation of the β1-adrenergic receptor-coupled CaMKII, p38 MAPK, and ATF6 pathway.

cardiomyocytes; signal transduction

EVIDENCE HAS ACCUMULATED indicating that autoimmunity plays an important role in viral myocarditis and dilated cardiomyopathy (18). Among the various known anti-heart autoantibodies, an antibody activating the human β1-adrenergic receptor (β1-AR) has been best studied. The autoantibody is not only present in 30–40% of patients with dilated cardiomyopathy (7, 20) but also has been shown to predict increased mortality in the patients (30). The importance of the β1-AR antibody in heart failure has been established by direct demonstration of dilated cardiomyopathy in animals immunized with a peptide corresponding to the sequence of the second extracellular loop of the β1-AR (β1-ECII) (8, 21). In addition, the β1-ECII antisera obtained from the experimental animals have been shown to be biologically active (28, 29), with a proapoptotic action via activation of the β1-AR on cultured cardiomyocytes (28). However, little is known about how the anti-β1-ECII antibody exerts its cardiodepressant effects distal to the β1-AR site or whether the β1-ECII-induced cardiomyopathy is affected by increased norepinephrine (NE). Since the failing myocardium is exposed to increased NE in heart failure patients, the potential interaction between NE and the anti-β1-ECII antibody on the β1-AR and its subsequent molecular and cellular pathways leading to cell death is of practical importance. In clinical studies, chronic adrenergic over-stimulation has been shown to increase mortality and left ventricular (LV) systolic dysfunction in patients with heart failure. In addition, since the cytokine and antibody production from antigen-specific CD4+ T and B lymphocytes is mediated via adrenergic receptors in the immune system (10), NE may also exaggerate the immune response in the animals to produce worsening of cardiomyopathy. Thus we speculate that NE may act additively or synergistically with the anti-β1-AR antibody to enhance the progression of cardiomyopathy.

Recently, our group (16, 17) reported that NE induces endoplasmic reticulum (ER) stress and cell apoptosis in cultured PC12 cells. Similar findings were observed in cultured neonatal rat cardiomyocytes and H9c2 cells (unpublished data). Furthermore, since β1-AR stimulation by NE is known to activate myocardial Ca2+/calmodulin-dependent protein kinase II (CaMKII) (38, 40–42, 44) and mitogen-activated protein kinases (MAPKs) (2–4, 11, 22, 24, 39) in the heart, we carried out the present studies in adult rabbits to determine whether the autoimmune cardiomyopathy induced by β1-ECII peptide is associated with activation of the β1-AR-coupled CAMKII-MAPK pathway and ER stress. We also carried out experiments in cultured neonatal rat cardiomyocytes to study the direct actions of the anti-β1-ECII antibody on the β1-AR-coupled CAMKII-MAPK pathway and ER stress, using the immunoglobulin (Ig)G obtained from the sera of β1-ECII peptide-immunized animals.

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METHODS

This study was approved by the University of Rochester Committee on Animal Resources and conformed to the guiding principles approved by the Council of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1996, Office of Science and Health Reports].

Animal immunization and NE treatment. Adult New Zealand White rabbits (2.8-4.3 kg) were immunized subcutaneously with a 26-amino acid β1-ECII peptide (residual 197-222; NH-HW-WRAESEDCRNYNDPSCCDVTRN-COOH; Bethyl Laboratories, Montgomery, TX). Animals were given either 1 mg of the β1-ECII peptide dissolved in 0.5 ml of saline conjugated with 0.5 ml of complete or incomplete Freund’s adjuvant or the Freund’s adjuvant (1 ml) alone once a month for 6 mo. Blood samples were collected from a central ear vein once a month for measuring the anti-β1-ECII antibody using an ELISA method. Development of cardiomyopathy was followed monthly with echocardiography. At month 6, animals were prepared for resting hemodynamic measurements and plasma NE assays and then killed for heart harvest for various tissue measurements.

To study the influence of NE on development of cardiomyopathy, rabbits with and without β1-ECII immunization were randomized to receive either two subcutaneous NE pellets (50-μg pellets, 90-day release) given 3 mo apart to keep the release of NE constant at ~0.5 mg/day or two placebo pellets (Innovative Research of America, Sarasota, FL) administered similarly. The NE dose was chosen after preliminary studies showed that larger doses caused early sudden death in cardiomyopathic animals.

ELISA for anti-β1-ECII antibody determination. β1-ECII peptide was used to coat a 96-well microtiter plate (Nunc, Roskilde, Denmark) overnight at 4°C. The wells were then saturated with a phosphate-buffered saline (PBS), pH 7.4, supplemented with 1% bovine serum albumin and 0.1% Tween 20. The rabbit plasma was added to the coated plates and incubated for 1 h at 37°C. Goat anti-rabbit IgG conjugated to horseradish peroxidase was then added. The optical density of color development after addition of 3,3′,5,5′-tetramethylbenzidine was read at 450 nm with an Opsys MR microplate reader (Dynex Technology, Chantilly, VA).

Echocardiography. Animals were lightly anesthetized with ketamine (10 mg/kg im) and midazolam (0.6 mg/kg im), and the anterior chest was shaved. Transthoracic M-mode and two-dimensional echocardiography was performed with an Acuson 128XP/10c echocardiographic system (Acuson Computed Sonography, Mountain View, CA) and recorded on a midpapillary-level LV parasternal short-axis view, using a 5-MHz broadband transducer. LV fractional shortening (% LV end-diastolic/H11011 -LV end-systolic/H11011) was measured from averages of data over 5 s.

After the hemodynamic study, the animal was killed, and the heart, lungs, and liver were removed and weighed. LV muscle blocks were cleansed and then either embedded immediately in paraffin or stored in liquid nitrogen for later analyses.

Cardiomyocyte apoptosis. Cardiomyocyte apoptosis was measured with both terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) detection and caspase-3 activity. The TUNEL detection was performed on paraffin-embedded LV tissue slides with an Apoptosis Detection System (Promega, Madison, WI), according to the manufacturer’s instructions. The slides were treated with terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP to produce TUNEL-positive cells. Sections also were stained with propidium iodide (Sigma-Aldrich, St. Louis, MO) and mouse anti-myosin heavy chain antibody (Chemicon International, Temecula, CA) to identify myocytes under an Olympus BX-FLA reflected light fluorescence microscope (Olympus Imaging America, Melville, NY). Apoptosis was calculated as the number of TUNEL-positive nuclei per 10,000 cardiomyocytes, determined by random counting of 14 fields per section.

Caspase-3 activity was determined with a caspase-3 colorimetric assay kit (R&D Systems, Minneapolis, MN), following the manufacturer’s instructions. Tissue lysate was incubated with caspase-3 colorimetric substrate (DEVD-pNA), and the release of chromophore pNA was read on the Opsys MR microplate reader at 405 nm. The data are expressed in arbitrary densitometry units.

Tissue immunocytochemistry. Antigen retrieval was obtained in deparaffinized LV tissue slides by heating in a Tris buffer with 0.1% Tween 20 plus EDTA (1 mM), pH 7, at 95 °C for 20 min. Nonspecific binding was blocked by 10% horse serum blocking buffer. The slides were then incubated with the following primary antibodies: monoclonal anti-caspase-12 antibody (1:50; Sigma-Aldrich), anti-C/EBP homologous protein (CHOP) antibody (1:100; Santa Cruz Biotechnologie, Santa Cruz, CA), and anti-glucose-regulated protein 78 (GRP78) antibody (1:100; BD Biosciences, St. Jose, CA), followed by mouse anti-mouse second antibody (1:500) and an Elite ABC Vectastain kit (Vector Laboratories, Burlingame, CA). Bound antibodies were detected using an Olympus BX40 microscope (Olympus Imaging America) and a Retiga 200R Fast1394 camera (Q-Imaging, Burnaby, BC, Canada).

Western blot analyses. Tissue protein was extracted in a lysis buffer for preparation of a whole cell lysate by centrifugation at 12,000 g for 4°C in 15 min and an ER membrane fraction by centrifugation at 100,000 g for 30 min. To prepare nuclear extracts, tissue homogenate was first centrifuged for 30 s at 2,000 rpm at 4°C, and the pellet was then resuspended in an ice-cold hypertonic salt buffer and centrifuged again for 15 min at 4°C. Protein concentration was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL).

For Western blotting, protein (~30-50 μg) was loaded onto 8-12% SDS-polyacrylamide gels and transferred electrically to a polyvinylidene difluoride membrane. Blots from whole cell lysates were probed with the following antibodies: anti-phospho-CaMKIIβ (Thr286) (1:500; Affinity BioReagents, Golden, CO), anti-CaMKIIβ (1:1,000; Santa Cruz), monoclonal mouse anti-CHOP (1:200; Santa Cruz), and anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-p38/p44 MAPK (Thr183/Tyr185), anti-p38 MAPK, anti-p44/42 MAPK, and anti-c-SAPK/JNK (all 1:1,000; Cell Signaling Technology, Danvers, MA). Blots from the ER membrane fraction were incubated with monoclonal anti-caspase-12 (1:500; Sigma-Aldrich) and anti-GRP78 (1:2,000; BD Biosciences). Anti-activating transcription factor 6 (ATF6) (1:1,000; Alexis Biochemicals, San Diego, CA) was used for detecting the full-length p90ATF6 and its cleaved forms in the nuclear fraction. A monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Imgenex, San Diego, CA) was used to confirm equal loading of protein. The protein bands were visualized using the ECL detection kit (Amersham Biosciences, Piscataway, NJ). The optical density of the bands was determined using NIH 1.6 Gel Image program, and the readings were normalized to a control sample in an arbitrary densitometry unit.

CaMKII kinase assays. CaMKII activity was measured using a CaMKII assay kit (Upstate), which is based on phosphorylation of a
specific substrate peptide (KKALRRQETVDAL) by the transfer of γ-phosphate of adenosine-5’-[32P]ATP by CaMKII. The final reaction mixture was spotted on the numbered P81 paper and used for scintillation counting. The results are expressed as picomoles of phosphorylated peptide per minute per 100 μg of protein.

Plasma NE assay. Arterial blood was collected into tubes containing reduced glutathione and centrifuged to separate the plasma, which was stored at −70°C for assay. Plasma NE was measured using a BAS plasma catecholamine kit as described previously (12).

Cultured neonatal rat cardiomyocyte experiments. Neonatal rat ventricular cardiomyocytes were cultured as described previously (37) with minor modifications. Briefly, cardiac ventricles, taken from 1- to 2-day-old Sprague-Dawley rat neonates, were gently minced and enzymatically dissociated using collagenase H (Worthington Biochemical, Lakewood, NJ). Cells were collected by centrifugation and plated at a density of 5 × 10^4 cells/cm² on a 60-mm dish in DMEM (Cellgro; Mediatech, Herndon, VA) containing 10% (vol/vol) fetal bovine serum and 1% penicillin-streptomycin for 24 h. Cytosine 1-β-D-arabinofuranoside (10 μM; Sigma) was added to retard the growth of contaminating fibroblasts.

To study the direct effects of the β1-ECII antibody on cardiomyocytes, we obtained purified IgG from the sera of β1-ECII-immunized animals by using Montage antibody purification kits containing spin columns prepacked with ProSep-G affinity chromatography media (Millipore, Billerica, MA) and added it to cultured cardiomyocytes. We found in preliminary experiments that the β1-ECII IgG produced an apoptotic effect in the cardiomyocytes, and the effect was dose dependent and easily reproducible at 200 μg/ml. Furthermore, to study whether the effects of anti-β1-ECII antibody were mediated via activation of CaMKII or p38 MAPK, we also applied the β1-ECII IgG to cells that had been pretreated with either 10 μM KN-93, a synthetic CaMKII inhibitor (Calbiochem Biosciences, La Jolla, CA), or 10 μM SB 203580, a specific p38 MAPK inhibitor (Calbiochem), for 20 min. Control and β1-ECII IgG (200 μg/ml) were then added into the culture medium for either 6 h for the CaMKII and p38 MAPK determinations or 48 h for the GRP78, CHOP, caspase-3, and caspase-12 studies. At the end of the experiment, cells were harvested and lysed with ice-cold lysis buffer. The cell lysates were centrifuged, and supernatants were subjected to electrophoresis on SDS-polyacrylamide gel (10% for GRP78, CHOP, caspase-12, p38 MAPK, and p38 MAPK, we also applied the β1-ECII antibody on cardiomyocytes.

Statistical analysis. Results are means ± SE. Experimental data were analyzed using the RS/I Research System (Bolt, Beraneck and Newman Software Products, Cambridge, MA) and SYSTAT 11 software (SPSS, Chicago, IL). The statistical significance of differences among the groups was analyzed using one- or two-way analysis of variance. Bonferroni simultaneous confidence intervals for all comparisons were used to determine the statistical significance of a difference between two groups. Repeated-measures analysis of variance for two grouping factors and one within-trial factor was used to determine the effects of β1-ECII immunization and NE on LV end-diastolic dimension and fractional shortening. Correlation analysis was used to determine the effect of anti-β1-ECII antibody on various parameters. Differences were considered statistically significant if P < 0.05.

RESULTS

Clinical characteristics and resting hemodynamics. Animals tolerated β1-ECII immunization and NE administration without significant distress. In the β1-ECII-immunized animals, blood anti-β1-ECII antibody increased linearly over time, from a barely detectable baseline value (0.14 ± 0.06 optical density units) to 57.20 ± 3.75 units (P < 0.001) at month 6. In contrast, sham animals that received no β1-ECII showed no significant changes in anti-β1-ECII antibody (from 0.10 ± 0.02 to 0.24 ± 0.06 units).

Table 1 shows that neither β1-ECII immunization nor NE pellet affected heart rate, aortic pressure, heart weight, or lung or liver weight. However, LV end-diastolic pressure was increased in the animals that received β1-ECII immunization. LV dP/dt showed a significant decrease in the β1-ECII-immunized rabbits, but this decrease was abolished by the coadministration of NE pellet. Plasma NE was increased in β1-ECII-immunized rabbits, and this was increased further in those animals that also received NE pellets.

The serial changes of LV diastolic dimension and fractional shortening in rabbits after β1-ECII immunization and/or NE pellet administration are shown in Fig. 1. Animals that received sham immunization and no NE pellet showed only a small increase in LV diastolic dimension but no effect on LV fractional shortening. β1-ECII immunization caused LV dilation and decline of LV fractional shortening, beginning at 1–2 mo of immunization. Addition of NE exaggerated LV dilation and decline of LV fractional shortening in β1-ECII-immunized rabbits.

Table 1. Effects of β1-ECII immunization and NE pellet in experimental animals

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 11)</th>
<th>NE (n = 7)</th>
<th>β1-ECII Immunization (n = 16)</th>
<th>β1-ECII + NE (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>3.8 ± 0.1</td>
<td>4.1 ± 0.2</td>
<td>4.0 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>263 ± 10</td>
<td>273 ± 13</td>
<td>230 ± 13</td>
<td>247 ± 12</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>95 ± 4</td>
<td>88 ± 7</td>
<td>89 ± 4</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>LV EDP, mmHg</td>
<td>5.2 ± 0.4</td>
<td>4.7 ± 0.6</td>
<td>15.0 ± 1.8*</td>
<td>10.5 ± 1.5*</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td>4.74 ± 0.39</td>
<td>5.339 ± 602</td>
<td>3.327 ± 402*</td>
<td>5.100 ± 654*</td>
</tr>
<tr>
<td>LV weight, g</td>
<td>5.41 ± 0.19</td>
<td>5.29 ± 0.23</td>
<td>5.29 ± 0.15</td>
<td>5.37 ± 0.20</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>12.3 ± 0.5</td>
<td>12.8 ± 1.1</td>
<td>14.0 ± 1.0</td>
<td>13.9 ± 0.7</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>89 ± 4</td>
<td>92 ± 6</td>
<td>81 ± 4</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>Plasma NE, pg/ml</td>
<td>71 ± 10</td>
<td>253 ± 38*</td>
<td>440 ± 50*</td>
<td>1101 ± 88*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of experiments. β1-ECII, second extracellular loop of the β1-adrenergic receptor; NE, norepinephrine; LV EDP, left ventricular end-diastolic pressure; LV dP/dt, rate of rise of LV pressure. *P < 0.05 compared with control sham immunization. †P < 0.05 compared with β1-ECII immunization.
Significant interactions exist between the effect of 2.95, P/H11021, or immunization. †

Fractional shortening decreased (rabbits. The high coefficients of determination (rabbits, produced an additive effect in animals. The magnitude of changes was greatest in the animals that received both β1-ECII immunization and NE pellet.

Myocyte apoptosis and caspase-3 activity. The decline of LV systolic function and LV dilation in the β1-ECII-immunized animals was associated with myocyte apoptosis, as shown by the increases of TUNEL-positive cardiomyocytes and caspase-3 activity (Fig. 2). In addition, NE pellet, which was sufficient to induce cardiac apoptosis in sham-immunized rabbits, produced an additive effect in β1-ECII-immunized rabbits.

Significant correlations exist among the plasma anti-β1-ECII titer against TUNEL-positive myocytes (r = 0.95), caspase-3 activity (r = 0.87), reduction of LV fractional shortening (r = 0.70), and LV dP/dt (r = 0.78) in the β1-ECII-immunized animals. The high coefficients of determination (r²) suggest that the increase of anti-β1-ECII antibody was responsible for 90% of changes in TUNEL-positive cardiomyocytes and 76% of changes in caspase-3 activity in the cardiomyopathic animals, but the coefficients of determination were smaller, accounting for only 49 and 60% of the changes in the reductions of LV fractional shortening and LV dP/dt, respectively. NE pellet did not enhance the production of anti-β1-ECII antibody in the immunized animals (66 ± 6 vs. 67 ± 5 optical density units) but caused a greater extent of cardiomyocyte apoptosis (88.7 ± 5.5 vs. 40.3 ± 4.3 TUNEL-positive cells per 10,000 cardiomyocytes, P < 0.001).

ER proteins. Immunohistochemistry shows that the ER-resident protein GRP78 and transcription factor CHOP were increased, whereas procaspase-12 was decreased, indicating increased processing of procaspase-12 to active caspase-12 in the animals treated with β1-ECII immunization and NE pellet (Fig. 3). Changes in the GRP-78, CHOP, and procaspase-12 proteins were also confirmed by Western blots (Fig. 4 and Table 2). In addition, the reduction of procaspase-12 was associated with an increase in cleaved caspase-12, consistent with the increased processing of procaspase-12 in the experimental animals. The magnitude of changes was greatest in the animals that received both β1-ECII immunization and NE pellet.

Caspase-3 activity and MAPK proteins. β1-ECII immunization and NE pellet increased CaMII phosphorylation and CaMII activity in LV (Fig. 5). In addition, activation of CaMII was associated with increased phosphorylation of p38 MAPK and SAPK/JNK and decreased phosphorylation of p44/42 MAPK (ERK1/2) (Fig. 6). Table 3 shows that the effects of β1-ECII immunization and NE on MAPKs were additive, since the greatest response was produced in the animals treated with both β1-ECII immunization and NE pellet.
p90ATF6 was reduced in the LV myocardium in animals treated with β1-ECII immunization, NE pellet, or both. The changes were associated with increases of two smaller cleaved forms of p90ATF6: p60ATF6 and p36ATF6 (Fig. 7, top). Nuclear p36ATF6 increased about twofold in the animals treated with either β1-ECII immunization or NE pellet but increase further (4-fold) in animals that received both β1-ECII immunization and NE pellet (Fig. 7, bottom).

Effects of β1-ECII IgG in cultured neonatal rat cardiomyocytes. Similar to the effects of β1-ECII immunization in intact animals, the IgG extracted from the β1-ECII-immunized rabbit serum increased phosphorylation of CaMKII and p38 MAPK (Fig. 8) and caused ER stress and cellular apoptosis, as evidenced by increased GRP78 and CHOP and cleavage of caspase-12 and caspase-3 (Figs. 9 and 10) in the cultured cardiomyocytes. To study whether the ER stress was caused by CaMKII or p38 MAPK activation, we also studied the effects of CaMKII phosphorylation produced by β1-ECII IgG after KN-93 and SB 203580 pretreatment. Figure 8 shows that KN-93 pretreatment abolished the increase of phosphorylation of both CaMKII and p38 MAPK produced by β1-ECII IgG, but SB 203580 prevented only the phosphorylation of p38 MAPK induced by β1-ECII IgG. Phosphorylation of CaMKII produced by β1-ECII IgG was unaffected by SB 203580. Also, unlike the differential effects on CaMKII phosphorylation, KN-93 and SB 203580 were both capable of blocking the effects of β1-ECII IgG on the increased protein expression of GRP78, CHOP, and cleaved forms of caspase-12 and caspase-3.

Table 2. Effects of β1-ECII immunization and NE pellet on endoplasmic reticulum proteins

<table>
<thead>
<tr>
<th></th>
<th>Sham Immunization</th>
<th>β1-ECII Immunization</th>
<th>β1-ECII + NE Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP78</td>
<td>1.00±0.17</td>
<td>2.40±0.30*</td>
<td>1.51±0.11* 3.89±0.65†</td>
</tr>
<tr>
<td>CHOP</td>
<td>1.00±0.11</td>
<td>1.55±0.10*</td>
<td>1.46±0.09* 2.30±0.15†</td>
</tr>
<tr>
<td>Procaspace-12</td>
<td>1.00±0.04</td>
<td>0.50±0.06*</td>
<td>0.56±0.04* 0.19±0.03†</td>
</tr>
<tr>
<td>Cleaved caspace-2</td>
<td>1.00±0.11</td>
<td>2.10±0.19*</td>
<td>1.55±0.13 4.87±0.36†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 in each group for glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and procaspace-12 and n = 6 in each group for cleaved caspace-12. *P < 0.05, compared to control sham immunization, and †P < 0.05, compared with β1-ECII immunization.

Fig. 3. Representative micrographs showing increased immunohistochemical staining of glucose-regulated protein 78 (GRP78) and anti-C/EBP homologous protein (CHOP) proteins and decrease of procaspace-12 protein expression in rabbit hearts following β1-ECII immunization, NE pellet, or both. Tissue was also stained with hematoxylin and eosin.

Fig. 4. Representative GRP78, CHOP, procaspace-12, and active cleaved caspace-12 immunoblots of LV myocardium in animals with sham immunization, β1-ECII immunization, NE pellet, and β1-ECII immunization plus NE pellet. GAPDH was used as loading control.
DISCUSSION

β₁-ECII immunization induced a dilated cardiomyopathy characterized by progressive LV dilation, reduction of systolic function, and cardiomyocyte apoptosis in rabbits. We further found that although NE pellet improved LV dP/dt in β₁-ECII-immunized animals, it was detrimental in the long run because it not only reduced LV fractional shortening but also furthered LV dilation and cardiomyocyte apoptosis. Thus our present study provides direct experimental evidence that adrenergic stimulation, although it may improve acute hemodynamics, is detrimental in chronic heart failure. Our findings also support the clinical use of β-receptor blockers, which have been shown to increase LV systolic function and reduce cardiovascular morbidity and mortality in heart failure subjects.

Results of our present study are consistent with prior studies using β₁-ECII immunization to produce experimental cardiomyopathy in rabbits (21) and rats (8). In rabbits, Matsui et al. (21) showed the effects of β₁-ECII were gradual and produced LV dilation and systolic dysfunction, but there was no clinically evident congestive heart failure until 12 mo of immunization. In our study, although the animals exhibited no gross pulmonary edema or liver enlargement after 6 mo of immunization, LV failure was evident as shown by the increases of LV end-diastolic dimension and pressure as well as the decline of LV dP/dt. LV weight did not increase significantly in our β₁-ECII-immunized animals. Iwata et al. (6) also showed no increase in LV weight in rabbits after 3 mo of β₁-ECII immunization, but there was a small (8%) increase in LV weight after 6 mo of immunization. A small increase (9%) in LV weight was also noted in rats after 9 mo of immunization. In contrast, a greater (21%) increase in LV weight occurred after 12 mo of immunization (21). Thus LV hypertension is not a striking feature in the early phase of the β₁-ECII peptide-induced cardiomyopathy. Clinical congestive failure and LV hypertrophy occur late in the disease process.

![Fig. 6. Protein expression of the phosphorylated and total MAPK family signals (p38 MAPK, p44/42 MAPK, and p54/46 SAPK/JNK). The results show that β₁-ECII and NE pellet increased phosphorylation of JNK and p38 MAPK but decreased phosphorylation of p44/42 MAPK. NE pellet potentiated the effects of β₁-ECII immunization on activation of the MAPK family proteins.](image)

![Fig. 7. Top: β₁-ECII immunization and NE pellet caused cleavage of p90ATF6 into 2 smaller fragments (p60ATF6 and p36ATF6), shown in the nuclear fraction. GAPDH was included as loading control. Bottom: nuclear p36ATF6 levels in 4 experimental groups. Values are means ± SE; n = 7–8. *P < 0.05 compared with control sham immunization. †P < 0.05 compared with β₁-ECII immunization.](image)

### Table 3. Effects of β₁-ECII immunization and NE pellet on MAPK family proteins

<table>
<thead>
<tr>
<th></th>
<th>Sham Immunization</th>
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<th>β₁-ECII Immunization</th>
<th>β₁-ECII + NE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NE</td>
<td>β₁-ECII</td>
<td>β₁-ECII + NE</td>
</tr>
<tr>
<td>p-p38 MAPK (p-p38/total p38)</td>
<td>0.24±0.02</td>
<td>0.69±0.17*</td>
<td>0.72±0.24*</td>
<td>2.70±0.46*†</td>
</tr>
<tr>
<td>p-ERK protein (p-ERK/total ERK)</td>
<td>1.36±0.13</td>
<td>0.96±0.14*</td>
<td>1.05±0.15*</td>
<td>0.48±0.07*†</td>
</tr>
<tr>
<td>p-JNK protein (p-JNK/total JNK)</td>
<td>0.60±0.11</td>
<td>1.10±0.10*</td>
<td>1.09±0.13*</td>
<td>1.27±0.11*</td>
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Values are means ± SE; n = 7–8 in each group. The ratios of phospho(p)-p44/42 MAPK (ERK1/2) to total ERK1/2, phospho-JNK to total JNK, and phospho-p38 MAPK to p38 MAPK were used to express the functional activity of the MAPKs. *P < 0.05 compared with control sham immunization. †P < 0.05 compared with β₁-ECII immunization.
In rats, Jahns et al. (8) reported that isogenic transfer of sera taken from rats immunized with \( \beta_1 \)-ECII to healthy inbred rats produced a dilated cardiomyopathy and myocyte apoptosis, as in donor animals. The findings indicate that the sera from the cardiomyopathic animals are biologically active and that the anti-\( \beta_1 \)-ECII antibody is directly responsible for the dilated cardiomyopathy, presumably via its partial agonistic action on the cardiac \( \beta_1 \)-AR. In our present study, NE pellet did not enhance the production of anti-\( \beta_1 \)-ECII antibody in the animals treated with \( \beta_1 \)-ECII immunization. The effects of \( \beta_1 \)-ECII immunization and NE pellet on cardiomyocyte apoptosis probably are additive, likely secondary to their respective effects on the cardiac \( \beta \)-ARs. The lack of effects of NE on immune activation after autoimmune stimulation is consistent with a recent finding that rat autoimmune myocarditis induced by cardiac myosin was suppressed by \( \beta_2 \)-adrenergic agonists and aggravated by propranolol, whereas the \( \beta_1 \)-selective blocker metoprolol had no effect (23).

ER stress was induced in both animals treated with NE pellet and \( \beta_1 \)-ECII immunization and cultured neonatal cardiomyocytes exposed to \( \beta_1 \)-ECII IgG. This was evidenced by the increases of GRP78 and CHOP and increased processing of procaspase-12 and ATF6. Results of our study suggest that the ER stress in autoimmune cardiomyopathy in animals was linked to activation of ATF6, a member of the ATF/CREB family of transcription factors (26, 27). ATF6 is a 670-amino acid ER-transmembrane protein with the NH\(_2\) terminus oriented toward the cytosol and the COOH terminus toward the ER lumen (1, 34). It exists in two full-length isoforms, p90ATF6\(\alpha\) and p110ATF6\(\beta\). When stimulated, ATF6 is translocated from the ER to the Golgi apparatus (1, 27), where the full-length ATF6s are cleaved by site-1 and site-2 proteases to several different NH\(_2\)-terminal active ATP6s. It is known that the smaller active ATP6s enter the nucleus and bind to the ER stress response elements to activate expression of several ER stress response genes, including GRP78. The increases in nuclear p60ATF6 and p36ATF6 (Fig. 7) in our present experiments are consistent with the activation and nuclear translocation of ATF6. ER stress also may be stimulated by underglycosylation of newly formed p90ATF6 (5).

The importance of ATF6 in ER stress is supported by the finding that the induction of GRP78 reporter genes was blocked by dominant negative forms of ATF6 in the presence of ER stress (13). Expression of ER stress-inducible genes was also reduced by knockdown of ATF6 using small interfering RNA technology. However, the upstream signals for ATF6 activation have not been fully established. \( \beta_1 \)-ECII is increased by ER Ca\(^{2+}\) depletion (5). In addition, p38 MAPK may serve as an upstream signal for ATF6 (34). Luo and Lee (13) showed that ATF6 is not only a substrate of p38 MAPK in a kinase assay but also can be phosphorylated by p38 MAPK. p38 MAPK has been shown to phosphorylate multiple ER stressors, such as ATF2 and CHOP, leading to an increase in transcription activity (35). The simultaneous increases of phospho-p38 MAPK and cleaved ATF6s suggest that these changes may be causally related, but additional studies are needed to establish their cause-and-effect relation.
Activation of CaMKII was evidenced by the increased CaMKII enzyme activity and phospho-CaMKII6 in the LV myocardium of β1-ECII-immunized and NE-treated animals. CaMKII6 phosphorylation also increased in cultured neonatal cardiomyocytes following β1-ECII IgG treatment. CaMKII is a critical downstream element of the β1-AR signal pathway. CaMKII6 is the predominant isoform in the heart (14). β1-AR stimulation increases cytosolic Ca2+ and free Ca2+/calmodulin concentrations in adult cardiac myocytes (15). CaMKII6 is also phosphorylated in the isolated ventricular cardiomyocytes by β1-AR stimulation; this change has been shown to induce fetal gene induction (31) and cardiac apoptosis (42). Overexpression of activated CaMKII6 or CaMKIV also has been shown to induce cardiac hypertrophy in transgenic mice (41), whereas CaMKII inhibitor KN-62 blocks endothelin-1-induced hypertrophy in cultured cardiomyocytes (43). CaMKII inhibition in a transgenic mouse model also has been shown to prevent cardiac remodeling after myocardial infarction (40). Results of our present study suggest that CaMKII plays an important role in p38 MAPK phosphorylation and subsequent ER stress activation in autoimmune cardiomyopathy. The findings in vivo are further supported by our experiments in cultured rat neonatal cardiomyocytes showing that inhibition of CaMKII activation by KN-93 prevented increased phosphorylation of p38 MAPK and ER stress-mediated caspase activation. KN-93 selectively binds to the calmodulin binding site of CaMKII. It acts to block the action of CaMKII by preventing the association of calmodulin with the enzyme. In addition, our results show that p38 MAPK inhibition by SB 203580, which had no effect on CaMKII phosphorylation, abolished the ER stress activation by β1-ECII IgG. The findings suggest that activation of CaMKII by β1-ECII IgG occurs before that of p38 MAPK. Our results are consistent with a report showing that CaMKII serves as an activator of p38 MAPK pathway in response to Ca2+ (32).

In addition to the increase of phospho-p38 MAPK, β1-ECII immunization activated the SAPK/JNK and depressed the ERK pathway. Similar changes in MAPKs have been shown to occur in pacing cardiomyopathy (25) and myocardial ischemia-reperfusion damage (22, 39). Activation of p38 MAPK in β1-ECII-induced cardiomyopathy is probably proapoptotic, because selective pharmacological inhibition of p38α MAPK has been shown to improve cardiac function and reduce myocyte apoptosis in a model of myocardial injury (11). This is also supported by our findings in cultured neonatal rat cardiomyocytes using the specific p38 MAPK inhibitor SB 203580. However, other studies have shown that p38 MAPK may exert an antiapoptotic effect in cardiomyocytes following β1-AR stimulation (2). Also, in a recent study of transgenic animals, Peter et al. (24) reported that inhibition of p38 MAPK rescues cardiomyopathy induced by overexpression of β2-AR but not β1-AR. The conflicting reports suggest that the functional role of p38 MAPK in cardiomyocytes may vary in different experimental conditions and/or animals. Studies also have shown that activation of JNK is linked to ER stress-mediated apoptosis and caspase-12 cleavage (33). Prolonged JNK activation has a proapoptotic effect under various cell death-inducing stimuli, such as oxidative stress and DNA damaging agents (4, 9). Also, using a highly specific peptide inhibitor of JNK, Milano et al. (22) showed that inhibition of JNK reduces myocardial ischemic-reperfusion injury and infarct size in anesthetized rats in vivo. On the other hand, ERK activation is antiapoptotic in most tissues (36). Finally, NE has a pro-oxidant action, which could be the mediator for many of the proapoptotic and antiapoptotic effects observed.

Although a direct cause-and-effect relationship between β1-AR activation and the downstream signal transduction was not established for the autoimmune cardiomyopathy in our present study, prior investigators (6, 19) have shown that the morphological cardiomyopathic changes are reduced by the β1-receptor blocker bisoprolol in the experimental animals. The β1-AR-mediated apoptotic effect of the β1-ECII antibodies also has been shown directly in adult isolated cardiomyocytes, using metoprolol pretreatment (28). Studies are now in progress in our laboratory to show whether the protective effects of metoprolol on cardiac dysfunction and myocyte

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**Fig. 10. Effects of β1-ECII IgG, KN-93, and SB 203580 on activation of GRP78 and CHOP and cleavage of procaspase-12 and caspase-3 (Western blots) in cultured neonatal rat cardiomyocytes. The optical density was normalized to a control sample in an arbitrary unit. Values are means ± SE; n = 6 in each group. *P < 0.05 compared with control. †P < 0.05 compared with β1-ECII IgG alone.**
apoptosis in the autoimmune cardiomyopathy are associated functionally with attenuation of the CaMKII, p38 MAPK, and ER stress signals.

In conclusion, we have provided new experimental evidence that autoimmune cardiomyopathy induced by β1-ECII peptide is associated with an increase of plasma NE and β1-AR-mediated stimulation of CaMKII, p38 MAPK, and ATF6 cleavage and nuclear translocation. Administration of NE does not promote immunologic response to β1-ECII immunization but produces additive effects on the activation of the β1-AR-coupled CaMKII-p38 MAPK-ATF6 pathway. Activation of ATF6 may then lead to ER stress, as evidenced by the upregulation of GRP78 and CHOP, increased cleavage of caspase 12, and, finally, myocyte apoptosis by caspase-3 activation. In addition, the increase of apoptotic JNK and decrease of antiapoptotic ERK may contribute to the cardiomyopathy. Further studies are needed to investigate the pathophysiological significance and complex interactions among the various apoptotic and antiapoptotic pathways in autoimmune cardiomyopathy.

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