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Pacing-Induced Heart Failure in Dogs Enhances the Expression of p53 and p53-Dependent Genes in Ventricular Myocytes

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Background—Rapid ventricular pacing in dogs is characterized by a dilated myopathy in which myocyte cell death by apoptosis may play a significant role in the impairment of cardiac pump function. However, the molecular mechanisms implicated in the modulation of programmed cell death under this setting remain to be identified. Moreover, questions have been raised on the specificity and sensitivity of the histochemical detection of DNA strand breaks in nuclei by the terminal deoxynucleotidyl transferase (TdT) reaction.

Methods and Results—Changes in the expression of Bcl-2 and Bax and their transcriptional regulator, p53, were determined by Western blot analysis in myocytes isolated from dogs affected by pacing-induced heart failure. A mobility shift assay for p53 binding activity was also performed. In addition, apoptosis was measured by confocal microscopy, which allowed the simultaneous detection of chromatin alterations and DNA damage. p53 DNA binding activity to the bax promoter was increased in nuclear extracts from myocytes obtained from failing hearts, and this response was associated with enhanced expression of Bax protein, 52%, and attenuation of Bcl-2, −92%. Immunolabeling of p53 in myocyte nuclei, measured by confocal microscopy, was 100% higher in cells from paced hearts. The combination of the TdT assay and confocal microscopy demonstrated that 20 myocyte nuclei per 10^6 were undergoing apoptosis in control myocardium and 4000 per 10^6 after pacing. Moreover, DNA laddering was shown in myocytes by agarose gel electrophoresis of DNA fragments.

Conclusions—The activation of p53 and p53-dependent genes may be critical in the modulation of myocyte apoptosis in pacing-induced heart failure. (Circulation. 1998;97:194-203.)

Key Words: molecular biology ■ heart failure ■ apoptosis ■ pacing

The cellular mechanisms implicated in the onset and progression of congestive heart failure (CHF) remain to be defined.1 Obscure are the pathogenetic events responsible for the initial adaptation and subsequent deterioration of cardiac performance when a chronic excessive circulatory load is imposed on the heart. The causative factor critical for this unfavorable progression is unknown, and animal models of the human disease are difficult to obtain. In this regard, pacing-induced heart failure in dogs has characteristics that closely mimic the alterations seen in idiopathic dilated cardiomyopathy in humans.2-5 Sustained ventricular pacing induces CHF and changes in cardiac anatomy, consisting of an increase in cavity diameter and thinning of the wall, that typically are found in the decompensated human heart. Although not all studies are in agreement,6,7 myocyte cellular hypertrophy and proliferation are both implicated in the dilated myopathy,8-10 but these cellular growth adaptations do not result in a significant increase in ventricular mass. Myocyte cell loss is a major component of pacing-induced CHF,9 and this phenom-

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generation may be crucial in stimulating apoptosis. Bcl-2 forms heterodimers with other members of the Bcl-2 family of proteins, such as Bax, which opposes the protective effects of Bcl-2, facilitating apoptosis. Importantly, the tumor suppressor gene p53 is a transcriptional regulator of the Bcl-2 and Bax genes. The actuation of p53 may increase the expression of Bax and decrease Bcl-2 in the cell, promoting apoptosis. Therefore, the induction of Bcl-2, Bax, and p53 in myocytes was evaluated in dogs affected by pacing-induced CHF to determine whether modifications in the expression of these genes were implicated in the modulation of myocyte cell death in this model. In addition, apoptosis was evaluated by confocal microscopy, which allowed the simultaneous detection of TdT labeling and chromatin alterations, and by agarose gel electrophoresis of DNA fragments from isolated myocytes.

Methods

Ventricular Function

Mongrel dogs weighing 18 to 20 kg were instrumented with catheters, probes, and a corkscrew electrode in the left ventricle attached to a portable external pacemaker (Pace Medical EV3434) as described previously. Briefly, each dog was sedated with acepromazine (1 mg/kg SC) and then anesthetized with sodium pentobarbital (25 mg/kg IV). A Tygon catheter (Cardiovascular Instruments) was placed in the descending thoracic aorta, and a solid-state manometer (Koningsberg P5.6) was inserted into the left ventricle through the apex under sterile surgical techniques. Dogs were allowed to recover from surgery for 7 to 10 days. Initial experiments were conducted when animals were awake and had trained to lie quietly without restraint on the laboratory table. Hearts were then paced at 210 bpm for 3 weeks and at 240 bpm for an additional week (n = 17). The control group was similarly instrumented but not paced (n = 19). Seven control and 5 paced dogs were used in previous studies. The protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the Guiding Principles for the Use and Care of Laboratory Animals of the American Physiological Society and the National Institutes of Health. Hemodynamic studies were performed with the dogs in a conscious state with the pacemaker turned off. Once the steady state was reached, the hemodynamic measurements were made. Heart rate, systemic arterial pressure, left ventricular systolic and end-diastolic pressures, and left ventricular dp/dt were obtained.

Myocyte Isolation

In a group of 8 control and 8 paced hearts, myocytes were enzymatically dissociated from the left ventricular free wall. At the end of the hemodynamic measurements, hearts were removed, a portion of the anterior aspect of the left ventricle was dissected free, and a large branch of the left anterior descending coronary artery was identified and cannulated for perfusion with collagenase. The solutions were supplements of modified commercial Eagle-Joklik MEM. HEPES-MEM contained (in mmol/L) NaCl 117, KCl 5.7, NaHCO3 4.4, KH2PO4 1.5, MgCl2 17, HEPES 21.1, and glucose 11.7, with amino acids and vitamins, 2 mmol/L L-glutamine, and 21 mM/L/mL insulin; pH was adjusted to 7.2 with NaOH. The osmolality of this solution is 292 mOsm. Resuspension medium was HEPES-MEM supplemented with 0.5% BSA, 0.3 mmol/L CaCl2, and 10 mmol/L taurocholate, adjusted to 292 mOsm. The cell isolation procedure consisted of three main steps: For low calcium perfusion, blood was washed and collagenase digestion (selected type II, Worthington Biochemical Corp) perfusion of the myocardium was carried out at 32°C with HEPES-MEM gassed with 85% O2/15% N2. For mechanical tissue dissociation, after the myocardium was removed from the cannula, the endomyocardium and epimyocardium were separated and minced. Collagenase-perfused tissue was subsequently shaken in resuspension medium containing creatine, collagenase, and 0.3 mmol/L CaCl2. Supernatant cell suspensions were washed and resuspended in resuspension medium. For separation of intact cells, intact cells were enriched by centrifugation, with the supernatant discarded. This procedure was repeated four to five times in each preparation to remove nonmyocytes, cell debris, and residual collagenase. Each centrifugation was performed at 3000 g for 3 minutes. Subsequently, ~106 cells were suspended in 10 mL isotonic Percoll (final concentration, 41% in resuspension medium) and centrifuged for 10 minutes at 34g. Intact cells were removed from the pellet and washed, and smears were made to control the purity of the preparation. Rectangular trypan blue, excluding cells, constituted nearly 80% of all myocytes. The average numbers of myocytes collected per gram of myocardium were 6×106 to 7×106 and 3×106 to 4×106 in control and paced hearts, respectively. The contributions of interstitial cells were assessed by counting 1000 cells in each left ventricle and then computing from these counts the respective fractions of myocytes and nonmyocytes encountered. Nonmyocytes accounted for 2% to 3% of the cell population.

DNA Gel Electrophoresis

To detect internucleosomal cleavage of the DNA, the presence of low-molecular-weight DNA fragments was determined in left ventricular myocytes isolated from control and paced hearts. Myocytes were fixed for 24 hours at ~20°C in 70% ethanol. Cells were then centrifuged at 800g for 5 minutes, and the ethanol was thoroughly removed. Pellets were resuspended in 40 mL of phosphate-citrate buffer, consisting of 192 parts of 0.2 mol/L Na2HPO4 and 8 parts of 0.1 mol/L citric acid (pH 7.8) at room temperature for 1 hour. Samples were centrifuged at 1000g for 5 minutes. The supernatant was transferred to new tubes and concentrated by vacuum in a SpeedVac concentrator (Savant Instruments Inc) for 15 minutes. A 3-mL aliquot of 0.25% Nonidet NP-40 (Sigma Chemical Co) in distilled water was then added, followed by 3 mL of a solution of RNase, 1 mg/mL, also in water. After 30 minutes of incubation at 37°C, 3 mL of a solution of proteinase K, 1 mg/mL (Boehringer Mannheim), was added, and the extract was incubated for an additional 1 hour at 37°C. Subsequently, 12 mL of loading buffer (0.25% bromophenol blue, 30% glycerol) was added, and samples were subjected to electrophoresis on 2% agarose gel containing 0.5 µg/mL ethidium bromide. The DNA in the gel was visualized under UV light. This analysis was performed in myocytes obtained from 3 control and 3 paced dogs.

Western Blot

Total proteins were extracted from ventricular myocytes isolated from 6 control and 6 paced hearts. Myocytes were suspended in 200 µL of lysis buffer (in mmol/L: Tris-HCl 50, pH 7.4, EDTA 5, pH 8.0; NaCl 250, NaF 25; Na3VO4 0.1; PMSF 0.1; and DTT 5, plus 0.1% Triton X-100), incubated on ice for 30 minutes, and spun down at 14000 rpm for 10 minutes. Protein concentration was measured by the Bio-Rad protein assay, and samples containing 50 to 100 µg of total proteins were mixed with loading buffer (20% glycerol, 3% SDS, 3% DTT, 10 mmol/L EDTA, and 0.05% bromophenol blue), boiled for 2 minutes, and loaded onto 10% SDS-polyacrylamide gel. Proteins were electrophoresed onto Trans-Blot nitrocellulose membranes (Bio-Rad), blocked with 5% powdered milk in TBST (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, 0.5% Tween 20) overnight at 4°C, and incubated with the primary antibodies mouse anti-p35 rabbit antibody (PAH2400), rabbit anti-human Bcl-2 antibody (ΔC1), and rabbit anti-human Bax antibody (P-19, all Santa Cruz Biotechnology) diluted 1:500 with TBST. Blots were subsequently washed in TBST and incubated with goat anti-rabbit and goat anti-mouse hors eradise peroxidase–conjugated antibodies (Santa Cruz Biotechnology) diluted 1:10 000 in TBST. Bound antibodies were detected with ECL detection reagents (Amersham) and quantified with the JAVA image analysis system (Jandel Scientific). In control reactions, nonimmune rabbit serum was used instead of primary antibodies for Bcl-2 and Bax. For p53, mouse anti-Brdu was used. In all experiments, control reactions were negative.

In Situ TdT Assay

Tissue sections were mounted on poly-L-lysine–coated slides (Sigma Chemical Co). After deparaffinization and dehydration, tissue sections...
were incubated in PBS containing 0.1% saponin and 1 mM/mL EGTA for 30 minutes. This procedure enhances the enzymatic incorporation of nucleotides. Sections were then washed repeatedly in PBS, and DNA strand breaks were detected as previously described.23,29,30 Specifically, sections were covered with 50 μL of staining solution containing 5 U TdT, 2.5 mM/L CoCl₂, 0.2 mM/L potassium cacodylate, 25 mM/L Tris-HCl, 0.25% BSA, and 0.5 mM/L dUTP, coupled to biotin via a 16-atom spacer arm (biotin-16-dUTP). These reagents were all from Boehringer Mannheim Biochemicals. Sections were incubated in this solution for 30 minutes at 37°C in a humidified chamber. After being rinsed in PBS, sections were incubated for 30 minutes at room temperature in a solution containing 4× concentrated SSC buffer and 5% (wt/vol) nonfat dry milk (Sigma). Finally, the staining solution, which contained 5 μg/mL of fluorescein-isothiocyanate–labeled ExtrAvidin (Sigma), 4× concentrated SSC buffer, 0.1% Triton X-100, and 5% nonfat dry milk, was applied for 30 minutes. The tissue was incubated at 37°C for 30 minutes with the primary antibody (clone 5C5, Sigma) diluted 1:20 in PBS containing 10% goat serum and subsequently with anti-mouse IgG TRITC-labeled antibody (Sigma), also diluted 1:30 in PBS, containing 10% goat serum. Sections were then stained with propidium iodide, 10 μg/mL, for 15 minutes to visualize nuclei. After this procedure, sections were rinsed in PBS and embedded in Vectashield (Vector Laboratories) mounting medium.

Confocal Microscopy

The number of myocyte nuclei in the myocardium labeled by dUTP was measured by the number of stained nuclei counted per unit area of tissue sections in the left ventricle. This was done by confocal microscopy (Bio-Rad MR-1000) to correlate chromatin alterations with the presence or absence of dUTP labeling. Sections were examined with an ×100 objective (NA 1.3). The number of labeled nuclei was recorded, and the distinction between myocytes and nonmyocytes was obtained by detection of α-sarcromeric actin. In addition, the number of unlabeled myocyte nuclei in the tissue was determined. When these data were combined with the estimations of dUTP-labeled myocyte nuclei per unit area of myocardium, the number of apoptotic myocyte nuclei per 10⁶ nuclei was computed. This analysis by confocal microscopy was done in 3 control and 5 paced hearts. The percentage of myocyte nuclei is detectable by the TdT assay in human, 29 rat,30 and sheep hearts and 240 myocyte nuclei in 4 paced hearts. The human p53 staining.

Immunohistochemical Localization of p53 by Confocal Microscopy

For the detection of p53 in the myocardium, frozen sections were fixed for 5 minutes at −20°C in a methanol-acetone 2:1 mixture. After a washing in PBS, samples were incubated with p53 antibody (clone 80, Transduction Laboratories) overnight at 4°C. FITC-labeled anti-mouse IgG diluted 1:50 in PBS was used as secondary antibody. After a washing in PBS, samples were incubated in p53 antibody (5 μg/mL, for 15 minutes to visualize nuclei. After this procedure, sections were rinsed in PBS and embedded in Vectashield (Vector Laboratories) mounting medium.

Molecular Basis of Apoptosis

We have shown previously that DNA damage in myocyte nuclei is detectable by the TdT assay in human,29 rat,30 and dog31 myocardium. However, questions have been raised about this histochemical technique, concerning the possibility of labeling of nonapoptotic nuclei and the potential overestimation of this form of myocyte cell death.11–13 Therefore, a combination of dUTP labeling and confocal microscopy was used to determine whether myocyte nuclei stained by dUTP concurrently showed morphological alterations in chromatin structure. Fig 1A through 1C provides such an example in p53 staining.

Data Collection and Analysis

Tissue samples were coded, and the code was broken at the end of the experiment. Results are presented as mean±SD computed from the measurements obtained from each dog. Autoradiograms and gels were analyzed densitometrically by an image analyzer (Jandel Scientific). Statistical significance for comparisons between two measurements was determined by the unpaired two-tailed Student’s t test. Values of P<.05 were considered significant. Because measurements presented were not obtained in all animals, n values for each parameter are listed in the text or the legend to each figure.

Results

Ventricular Function

The modality of ventricular pacing used in the present study produced a severe impairment in cardiac pump function. Heart rate increased 33% (P<.001), from 94±21 bpm in controls (n=13) to 125±14 bpm in paced hearts (n=13). Moreover, mean arterial pressure was reduced 20% (P<.001), from 106±7 to 85±11 mm Hg; left ventricular systolic pressure 24% (P<.001), from 133±9 to 101±11 mm Hg; and left ventricular positive dP/dt 50% (P<.001), from 2815±303 to 1396±237 mm Hg/s. In contrast, left ventricular end-diastolic pressure increased 4.5-fold (P<.001), from 5.5±1.9 to 25±3.4 mm Hg. These measurements of ventricular performance were all collected in dogs in a conscious state and with the pacemaker turned off. Dogs subjected to ventricular pacing showed cachexia, tachypnea, ascites, pulmonary congestion, and pleural effusion. In summary, CHF occurred as a result of ventricular pacing.

Detection of DNA Strand Breaks in Myocyte Nuclei

We have shown previously that DNA damage in myocyte nuclei is detectable by the TdT assay in human,29 rat,30 and dog31 myocardium. However, questions have been raised about this histochemical technique, concerning the possibility of labeling of nonapoptotic nuclei and the potential overestimation of this form of myocyte cell death.11–13 Therefore, a combination of dUTP labeling and confocal microscopy was used to determine whether myocyte nuclei stained by dUTP concurrently showed morphological alterations in chromatin structure. Fig 1A through 1C provides such an example in p53 staining.
margination. Nuclear fragmentation was observed frequently, and this morphological pattern of apoptosis consistently showed dUTP staining (Fig 1D through 1F). At times, dUTP labeling of myocyte nuclei was not associated with apparent modifications in chromatin structure. In only one instance was nuclear damage detected in the absence of dUTP staining. In view of these results, the relative proportions of myocyte nuclei labeled by dUTP with and without chromatin changes were evaluated by confocal microscopy. This analysis included a total of 261 myocyte nuclei collected from the left ventricle of 6 paced dogs. The accumulated data indicated that 86.6±8.1% of dUTP-positive myocyte nuclei had alterations in chromatin structure, whereas 13±9% showed only dUTP staining. Moreover, 0.4±1.2% of damaged nuclei were not labeled by dUTP. These differences analyzed by χ² analysis were statistically significant (P<.005). Because DNA strand breaks precede the appearance of morphological changes typical of apoptosis,13 13% of TdT-positive cells most likely reflected early phases of cell death. This assumption was used in the evaluation of the magnitude of apoptosis by confocal microscopy in control and experimental animals. Heart failure resulted in a 201-fold (P<.001) increase in myocyte apoptosis from 20±9 per 10⁶ cells in sham-operated dogs (n=3) to 4020±960 per 10⁶ cells in paced animals (n=5). There was no correlation between the estimates of apoptosis and the functional measurements obtained in the decompenated hearts. In summary, ventricular pacing markedly increased programmed myocyte cell death.

DNA Laddering
The previous evaluation of DNA damage in pacing-induced heart failure was based on agarose gel electrophoresis of DNA extracted from myocardial tissue.10 This approach limited the interpretation of the results because it was not possible to discriminate whether DNA fragmentation was restricted to myocytes or involved interstitial fibroblasts and endothelial cells as well. To overcome this difficulty, pure preparations of myocytes from 3 control and 3 paced hearts were used to document DNA fragments in this cell population. Fig 2 illustrates that DNA fragments of size equivalent to the mononucleosomes and oligonucleosomes were detected in myocyte samples from failing hearts. This pattern of DNA damage was barely visible in control myocytes. In summary, pacing-induced heart failure was associated with DNA laddering in myocytes.

Figure 1. A, Fluorescence of propidium iodide staining shows apoptotic nucleus (arrow) and two nuclei with normal structure (arrowheads). dUTP labeling of apoptotic nucleus in A is shown in B by green fluorescence. C, Combination of propidium iodide and dUTP labeling in association with red fluorescence of α-sarcomeric actin staining of myocyte cytoplasm. Visualization of α-sarcomeric actin labeling required an increase in gain of photomultiplier, which resulted in overexposure of propidium iodide staining of nuclei in C. D, E, and F, depict, in a comparable manner, a myocyte with nuclear fragmentation. D, Propidium iodide and α-sarcomeric actin stainings; E, dUTP labeling by green fluorescence; F, three stainings together. Confocal microscopy: A–F, magnification ×1800.
Expression of Bcl-2 and Bax in Myocytes

Fig 3 illustrates the detection of Bcl-2 protein by Western blot in ventricular myocytes isolated from control and paced dogs. A 29-kD protein, Bcl-2, was apparent in myocytes collected from sham-operated animals. Conversely, this protein was barely visible in cells obtained from paced hearts. Densitometrically, in comparison with myocytes isolated from control animals (OD, 37±9; n=4), a 92% (P<.001) reduction (OD, 3±3; n=4) in Bcl-2 protein was measured in cells from dogs with heart failure. Changes in the opposite direction were observed with respect to the quantity of Bax protein in myocytes after pacing. As shown in Fig 4, the expression of Bax, a 21-kD protein, was upregulated in cells from failing hearts. Under these conditions, this cytoplasmic protein increased 52% (control: OD, 52±5, n=4; paced: OD, 79±13, n=4), and this difference was statistically significant (P<.005). In summary, pacing-induced cardiac failure was characterized by a decrease in the ratio of Bcl-2 to Bax protein in myocytes.

Expression, Localization, and Binding Activity of p53 in Myocytes

The consequences of pacing-induced heart failure on the expression of the tumor suppressor gene p53 were examined by Western blot (Fig 5). The quantity of this nuclear protein increased in myocytes collected from paced hearts (control: 15±8, n=4; paced: 71±12, n=4), and this 4.8-fold change was statistically significant (P<.001). A segment on the bax promoter containing a 39-bp sequence, from −486 to −448 bp, corresponding to the p53-binding site consensus motif24 was radiolabeled at its ends and used as a probe in a gel mobility shift analysis. This probe contained one perfect and three imperfect consensus sites for p53 binding.24 When nuclear extracts from myocytes collected from control and paced hearts were incubated with the radioactive probe, two complexes with shifted gel mobility were detected. However, the optical density of the p53 shifted bands was markedly increased in the preparations corresponding to failing hearts (Fig 6). Densitometrically, the optical density of the combined shifted complexes was 5±1 (n=4) in nonpaced dogs and 36±18 (n=4) in paced animals. This difference was statistically significant.
The specificity of this assay was confirmed by exposing nuclear extracts to unlabeled \textit{bax} in its original and mutated forms. In the first case, the shifted bands were no longer apparent, whereas in the second, the bands remained essentially intact (Fig 6). In addition, antibodies against p53 were included in some experiments to confirm the interaction of p53 with target DNA. Under these conditions, the shifted bands were abolished by the antibody (Fig 7). In contrast, the inclusion of irrelevant antibody did not modify the pattern of p53 DNA binding in myocytes from paced dogs. Moreover, the positions of the p53 shifted bands in SV-T2 cells were identical to those observed in myocytes. The formation of shifted complexes in SV-T2 cells was inhibited when p53 antibody was added to the assay.

To document further the activation of p53 and its translocation to the nucleus, the presence of this protein in myocyte nuclei was demonstrated morphologically by confocal microscopy (Fig 8). This approach allowed the quantitative analysis of the number of p53-labeled nuclei in control and paced dogs. Heart failure was characterized by 38\% (n=4) p53-labeled myocyte nuclei in the left ventricular myocardium, whereas 19\% (n=4) myocyte nuclei showed p53 staining in control tissue. This twofold difference was statistically significant (P<.02). In summary, ventricular pacing resulted in an upregulation and activation of p53 in myocytes that affected the response of the Bax gene.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5}
\caption{Western blot of p53 protein in normal and paced hearts. Equal loading of proteins is illustrated by Coomassie blue staining.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Gel mobility shift assay showing interaction of p53 with its consensus motif on \textit{bax} promoter. Nuclear extracts were obtained from myocytes collected from normal and paced hearts. p53-specific bands were subject to competition with an excess of unlabeled self-oligonucleotide competitor but not with unlabeled mutated form of \textit{bax} noncompetitor. Labeled \textit{bax} probe not incubated with nuclear extracts was included in assay to detect unspecific bands. Nuclear extracts from SV-T2 cells were used as a positive control for positions of shifted complexes. Arrows indicate positions of p53 shifted bands.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7}
\caption{Specificity of p53 shifted complexes in myocytes from pacing dogs was tested by incubating nuclear extracts with p53 antibody and irrelevant antibody. Nuclear extracts from SV-T2 cells were also incubated with p53 antibody and irrelevant antibody. Arrows indicate positions of p53 shifted bands. See Fig 6 for other symbols.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8}
\caption{Western blot of p53 protein in normal and paced hearts. Equal loading of proteins is illustrated by Coomassie blue staining.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure9}
\caption{Gel mobility shift assay showing interaction of p53 with its consensus motif on \textit{bax} promoter. Nuclear extracts were obtained from myocytes collected from normal and paced hearts. p53-specific bands were subject to competition with an excess of unlabeled self-oligonucleotide competitor but not with unlabeled mutated form of \textit{bax} noncompetitor. Labeled \textit{bax} probe not incubated with nuclear extracts was included in assay to detect unspecific bands. Nuclear extracts from SV-T2 cells were used as a positive control for positions of shifted complexes. Arrows indicate positions of p53 shifted bands.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure10}
\caption{Specificity of p53 shifted complexes in myocytes from pacing dogs was tested by incubating nuclear extracts with p53 antibody and irrelevant antibody. Nuclear extracts from SV-T2 cells were also incubated with p53 antibody and irrelevant antibody. Arrows indicate positions of p53 shifted bands. See Fig 6 for other symbols.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure11}
\caption{Western blot of p53 protein in normal and paced hearts. Equal loading of proteins is illustrated by Coomassie blue staining.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure12}
\caption{Gel mobility shift assay showing interaction of p53 with its consensus motif on \textit{bax} promoter. Nuclear extracts were obtained from myocytes collected from normal and paced hearts. p53-specific bands were subject to competition with an excess of unlabeled self-oligonucleotide competitor but not with unlabeled mutated form of \textit{bax} noncompetitor. Labeled \textit{bax} probe not incubated with nuclear extracts was included in assay to detect unspecific bands. Nuclear extracts from SV-T2 cells were used as a positive control for positions of shifted complexes. Arrows indicate positions of p53 shifted bands.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure13}
\caption{Specificity of p53 shifted complexes in myocytes from pacing dogs was tested by incubating nuclear extracts with p53 antibody and irrelevant antibody. Nuclear extracts from SV-T2 cells were also incubated with p53 antibody and irrelevant antibody. Arrows indicate positions of p53 shifted bands. See Fig 6 for other symbols.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure14}
\caption{Western blot of p53 protein in normal and paced hearts. Equal loading of proteins is illustrated by Coomassie blue staining.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure15}
\caption{Gel mobility shift assay showing interaction of p53 with its consensus motif on \textit{bax} promoter. Nuclear extracts were obtained from myocytes collected from normal and paced hearts. p53-specific bands were subject to competition with an excess of unlabeled self-oligonucleotide competitor but not with unlabeled mutated form of \textit{bax} noncompetitor. Labeled \textit{bax} probe not incubated with nuclear extracts was included in assay to detect unspecific bands. Nuclear extracts from SV-T2 cells were used as a positive control for positions of shifted complexes. Arrows indicate positions of p53 shifted bands.}
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\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure16}
\caption{Specificity of p53 shifted complexes in myocytes from pacing dogs was tested by incubating nuclear extracts with p53 antibody and irrelevant antibody. Nuclear extracts from SV-T2 cells were also incubated with p53 antibody and irrelevant antibody. Arrows indicate positions of p53 shifted bands. See Fig 6 for other symbols.}
\end{figure}

\section*{Discussion}

The results of the present study indicate that the tumor suppressor protein p53 was activated in pacing-induced CHF. The stimulation of this gene was coupled with the enhanced expression of Bax and the downregulation of Bcl-2 in myocytes. Although the relationship between the induction of p53 and its dependent genes and the initiation and progression of ventricular decompensation was not analyzed here, an increased p53 DNA binding activity was detected in the failing heart, suggesting that this transcription factor was implicated in myocyte apoptosis during the late stages of the cardiac myopathy. Such a contention was strengthened by the nuclear localization of p53 and by changes in
Bcl-2 and Bax levels in myocytes. The p53-mediated alteration in the relative proportion of Bcl-2 and Bax protein may be critical for the initiation of the suicide program of myocytes in response to death stimuli in CHF.

Pacing-Induced Heart Failure and p53
The anti-oncogene p53 has several functions, including the activation of transcription when it binds to specific DNA sequences in the promoter of several genes.24,34 p53 is involved in apoptosis,35,36 and it interferes with the cell cycle at the G1-S boundary37 and in the M phase.38 These inhibitory effects of p53 on the cell cycle are distinct from its influence on programmed cell death.39,40 In the presence of DNA damage, p53 may trigger apoptosis by suppressing cell cycle progression through the activation of WAF141 or by demanding DNA repair through the stimulation of GADD45.42 Such properties of p53 are relevant to cardiac failure, because cell death and proliferation both contribute to ventricular remodeling in the decompensated heart after pacing.10 Whether the initiation of apoptosis in this form of dilated myopathy is strictly related to the reentry of myocytes into the cell cycle or is mediated by other mechanisms cannot be established in the present investigation.

On the basis of in vitro observations, the enhanced expression and activation of p53 may not be sufficient to initiate apoptosis.24 However, p53 upregulates the transcription of bax and attenuates the induction of bcl-2,25 facilitating apoptosis. The promoter of bax contains one perfect and three imperfect consensus sites for p53 binding, and the activation of p53 may stimulate the bax gene. Conversely, a p53-negative response

Figure 8. Confocal microscopic images illustrating p53 localization in myocyte nucleus from paced dog. Myocytes were stained by α-sarcomeric actin and nuclei by propidium iodide (A). p53 antibody labeling of nuclei (arrows) is shown by green fluorescence (B). Three stainings are combined in C. Another example of p53 labeling of two myocyte nuclei (arrows) from paced dog is documented in D by green fluorescence. p53, α-sarcomeric actin, and propidium iodide staining are presented together in E. F corresponds to A431 human epidermoid carcinoma cells, which were used as positive control. Green fluorescence illustrates p53 staining in nuclei only. This is apparent by comparing F with G, which depicts same microscopic field by phase contrast microscopy. Magnification: A-E, ×2000; F and G, ×1000.
element has been identified in the \textit{bcl-2} gene,\textsuperscript{35} and this p53-dependent repression may attenuate \textit{bcl-2} transcription. Under these conditions, cells are more susceptible to apoptotic stimuli, which may be present in the diseased heart, as documented here with rapid ventricular pacing. It should be acknowledged that the factors responsible for p53 activation in myocytes after pacing are currently unknown. This phenomenon may involve phosphorylation of the p53 C-terminal regulatory domain by protein kinase C and casein kinase II,\textsuperscript{44} which may be enhanced by ligand binding of surface receptors in the decompensated heart. Possible candidates include the AT\textsubscript{1}, angiotensin II receptor subtype and \(\alpha\)-adrenergic receptors, which can be upregulated in the presence of ventricular dysfunction and failure.\textsuperscript{45,46}

We were unable to document that the p53 bands with shifted gel mobility could be further retarded by the addition of p53 antibodies in nuclear extracts from myocytes of normal and paced hearts. This may be a consequence of the level of expression of endogenous p53 in control and stressed myocytes. When p53 is overexpressed artificially in cell lines, the inclusion of p53 antibodies results in the generation of super-shifted complexes.\textsuperscript{24} Adult myocytes infected with a replication-deficient adenoviral vector containing wild-type human p53 also are characterized by p53 shifted and supershifted bands.\textsuperscript{47} Although a similar interaction between p53 and p53 antibodies could not be demonstrated here, p53 antibodies markedly attenuated the intensity of the p53 shifted complexes, confirming the specificity of the assay. Importantly, confocal microscopy showed a twofold increase in the fraction of myocyte nuclei labeled by p53 after pacing.

**Pacing-Induced Heart Failure and \textit{Bcl-2} and \textit{Bax}**

The \textit{Bcl-2} family of proteins contains several members that can inhibit or potentiate programmed cell death.\textsuperscript{48} These two opposite effects are the consequence of changes in the amount of proteins promoting cell survival, such as \textit{Bcl-2}, or facilitating the activation of the endogenous cell death pathway, such as \textit{Bax}.\textsuperscript{23} \textit{Bax} forms heterodimers with \textit{Bcl-2}, degrading its protective influence on cell viability; if \textit{Bax} homodimers predominate, cells are more disposed to undergo apoptosis.\textsuperscript{25} The mechanism by which \textit{Bcl-2} decreases the sensitivity of cells to die seems to be multifactorial and may include the regulation of Ca\textsuperscript{2+} homeostasis,\textsuperscript{49} attenuation of superoxide anion generation,\textsuperscript{18} interference with the stimulation of proteases of the interleukin-1\textsubscript{\beta}–converting enzyme family,\textsuperscript{50} and stabilization of mitochondrial membrane permeability.\textsuperscript{51,52} These functions of \textit{Bcl-2} are dependent on its state of phosphorylation.\textsuperscript{20–22} An increase in the quantity of \textit{Bax} and a decrease in \textit{Bcl-2} in the cell can be expected to diminish these various actions of \textit{Bcl-2} on cell survival, favoring the impact of death stimuli. The condition observed here after rapid ventricular pacing characteristically showed a reduction in the ratio of \textit{Bcl-2} to \textit{Bax} protein in myocytes and enhanced apoptosis. However, the members of the \textit{Bcl-2} family of proteins comprise not only \textit{Bcl-2} and \textit{Bax}, but also \textit{Bcl-x} in its long and short forms, \textit{Bak}, \textit{Bad}, and \textit{Mcl-1},\textsuperscript{32–35} which were not evaluated in the present study. Importantly, the several components of the \textit{Bcl-2} family can affect the susceptibility of cells to die, but cannot per se induce apoptosis in the absence of death stimuli.\textsuperscript{24} This applies to p53 as well.\textsuperscript{24} A recent report suggested that p53 may upregulate the myocyte renin–angiotensin system, which, in combination with a decrease in the ratio of \textit{Bcl-2} to \textit{Bax}, leads to myocyte cell death via the activation of surface AT\textsubscript{1} receptors.\textsuperscript{47} However, it is unknown whether a similar effector pathway is implicated in myocyte apoptosis after ventricular pacing. Other possibilities may involve physical forces generated by the increase of end-diastolic pressure in the decompensated heart\textsuperscript{9,30} and/or local ischemia associated with alterations in coronary blood flow.\textsuperscript{14} Loss of nitric oxide in the coronary endothelium with increased oxygen consumption\textsuperscript{14,17} occurs in this animal model, and this phenomenon may enhance the production of reactive oxygen species triggering apoptosis.

**Pacing-Induced Heart Failure and Myocyte Apoptosis**

In the past decade, several studies have documented that myocyte cell loss is a critical factor in the development and progression of ventricular dysfunction and failure.\textsuperscript{5,9,56,57} However, only recently, programmed cell death has been recognized as a relevant component of the diseased heart.\textsuperscript{1} Measurements of this process have been variable,\textsuperscript{10,26,30,58–61} raising questions on the specificity and sensitivity of the histochemical detection of DNA strand breaks in myocyte nuclei. In the present study, the occurrence of apoptosis in myocytes was demonstrated qualitatively by DNA agarose gel electrophoresis of isolated myocyte preparations and quantitatively by confocal microscopy. The latter approach showed that 86.6% of nuclei had chromatin modifications and positive TdT reaction, and 13% were labeled in the absence of morphological alterations in chromatin structure. Because double-strand cleavage of the DNA appears before changes in the morphology of the nucleus can be detected,\textsuperscript{33} the TdT assay resulted in an appropriate estimation of the degree of apoptosis after pacing. The mechanism by which the TdT assay was unable to stain 0.4% (1 of 261) of nuclei in advanced stages of chromatin fragmentation is difficult to explain. However, this may be the result of the generation of large DNA fragments of 50 to 300 kb during apoptosis without the formation of mononucleosomes and oligonucleosomes.\textsuperscript{62} Such a phenomenon has previously been observed in hepatocytes and epithelial and endothelial cells.\textsuperscript{62,63} Finally, it should be indicated that chronic ventricular pacing is characterized by fibroblast proliferation and myocardial scarring, which may have influenced the lower yields in myocyte isolation obtained in failing hearts. In addition, the greater degree of cell death in this group may have contributed to a reduction in the number of viable myocytes during collagenase digestion. These factors have to be considered in the interpretation of the results.

In conclusion, rapid ventricular pacing is characterized by the activation of the tumor suppressor protein p53, which may be implicated in the downregulation of \textit{Bcl-2} and the upregulation of \textit{Bax} in myocytes. This modification in antagonists and agonists of the endogenous cell-death pathway may represent the molecular counterpart of the increased susceptibility of myocytes to apoptotic stimuli in the failing heart. However, the nature of the death signal remains to be identified.
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Pacing-Induced Heart Failure in Dogs Enhances the Expression of p53 and p53-Dependent Genes in Ventricular Myocytes

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