Inhibition of Cardiac Myocyte Apoptosis Improves Cardiac Function and Abolishes Mortality in the Peripartum Cardiomyopathy of Gaq Transgenic Mice

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Background—Although the occurrence of cardiac myocyte apoptosis during heart failure has been documented, its importance in pathogenesis is unknown. Transgenic mice with cardiac-restricted overexpression of Gaq exhibit a lethal, peripartum cardiomyopathy accompanied by apoptosis. To test whether apoptosis is causally linked to heart failure, we assessed whether inhibiting this cell death would improve left ventricular function and survival in the Gaq peripartum cardiomyopathy model.

Methods and Results—The potent polycaspase inhibitor IDN-1965 or vehicle was administered subcutaneously to Gaq mice by osmotic minipump beginning on day 12 of pregnancy and continuing through euthanasia at day 14 postpartum. As expected, IDN-1965 markedly suppressed cardiac caspase-3–like activity (86.5%; \( P < 0.01 \)), accompanied by reduction in the frequency of cardiac myocyte apoptosis from 1.9 ± 0.3% to 0.2 ± 0.1% (\( P < 0.01 \)). Animals receiving IDN-1965 exhibited significant improvements in left ventricular end-diastolic dimension (vehicle, 4.7 ± 0.1 mm; IDN-1965, 4.2 ± 0.1 mm; \( P < 0.01 \)), fractional shortening (vehicle, 30.7 ± 1.2%; IDN-1965, 38.9 ± 1.0%; \( P < 0.01 \)), positive (vehicle, 3972 ± 412; IDN-1965, 5870 ± 295; \( P < 0.01 \)) and negative (vehicle, 2365 ± 213; IDN-1965, 3413 ± 201; \( P < 0.01 \)) dP/dt, and complete suppression of mortality (vehicle, 6 of 20 died; IDN-1965, 0 of 14 died; \( P < 0.05 \)).

Conclusions—Reduction in cardiac myocyte apoptosis by caspase inhibition improved left ventricular function and survival in pregnant Gaq mice. These data indicate that cardiac myocyte apoptosis plays a causal role in the pathogenesis of cardiomyopathy in this model. Caspase inhibition may provide a novel therapeutic target for heart failure. (Circulation. 2003;108:3036-3041.)

Key Words: apoptosis ■ caspases ■ cardiomyopathy

The pathogenesis of dilated cardiomyopathy is poorly understood. Although abnormalities in myocyte energetics, \(^1\) calcium handling, \(^2,3\) cytoskeleton, \(^4\) and adrenergic signaling \(^5\) have been implicated, the precise molecular events that mediate this complex syndrome are not known. Recently, cardiac myocytes have been noted to undergo apoptosis in various animal models of heart failure \(^6-17\) as well as in failing human hearts. \(^18-21\) The frequency of these cell deaths in human cardiomyopathy specimens is quite low (0.08% to 0.25%), \(^19-21\) although markedly higher than in controls (0.001% to 0.01%). Whether the cumulative effects of cardiac myocyte apoptosis contribute to the pathogenesis of heart failure, are an epiphenomenon, or are even a protective mechanism has not been determined.
Apoptosis in all metazoan cells is mediated in part by a family of aspartate-specific cysteine proteases called caspases.\textsuperscript{25} Caspase activation is brought about by two overlapping central death pathways: one involving cell surface receptors\textsuperscript{26} and the other mitochondria.\textsuperscript{27} Both the death receptor\textsuperscript{28,29} and mitochondrial pathways\textsuperscript{30–34} have been shown to be important for apoptosis in cardiac myocytes. The mitochondrial pathway plays a key role in mediating apoptosis in response to Gaq overexpression,\textsuperscript{35} in which it has recently been shown to be activated through the transcriptional upregulation of Nix/Bnip3L, a BH3-only proapoptotic Bcl-2 protein.\textsuperscript{36}

In the present study, the Gaq peripartum cardiomyopathy model was used to test the hypothesis that cardiac myocyte apoptosis is a mechanistic component of heart failure. To achieve this, we reduced cardiac myocyte death markedly during pregnancy in Gaq mice by chronic administration of a caspase inhibitor and then assessed whether the expected onset of cardiomyopathy and organisal death was ameliorated. The results indicate that cardiac myocyte apoptosis is indeed causally linked to the pathogenesis of lethal dilated cardiomyopathy in this model.

**Methods**

**Gaq Transgenic Mice**

Construction and characterization of the Gaq transgenic mice, which overexpress mouse Gaq exclusively in the myocardium under the control of the mouse \( \alpha \)-cardiac myosin heavy chain promoter, has been described elsewhere.\textsuperscript{23} The present study used the line containing 40 copies of the transgene on an FVB/N background, resulting in expression of the transgene protein at levels 5-fold higher than the endogenous protein.

**Treatment Protocol**

The protocol was approved by the Institute of Animal Studies of the Albert Einstein College of Medicine. Ten-week-old, pregnant Gaq transgenic mice bred in our laboratory (n = 34) were divided into 2 groups that received either vehicle (n = 20) or IDN-1965 (n = 14). Vehicle consisted of 50% DMSO, and the IDN-1965 solution consisted of 60 \( \mu \)g/\( \mu \)L of the compound in 50% DMSO. Each infusion was administered by subcutaneous osmotic minipump (Alzet model 1002, Alza Corp) implanted into the back as described by the manufacturer, with a delivery rate of 0.25 \( \mu \)L/h starting on day 12 of pregnancy and continuing through postpartum day 14. This infusion volume delivered 15 \( \mu \)g/h of IDN-1965. Because each osmotic minipump contained only 100 \( \mu \)L, pumps were replaced on postpartum day 3. On postpartum day 14, animals underwent echocardiography and left ventricular (LV) catheterization as described previously.\textsuperscript{37} The operators were blinded to the treatment group.

**Echocardiography and Cardiac Hemodynamics**

Cardiac function and hemodynamics were assessed by transthoracic echocardiography and left ventricular (LV) catheterization as described previously.\textsuperscript{37} The operators were blinded to the treatment group.

**Tissue Collection**

The ventricular portion of the heart was cut perpendicular to the long axis into 2 portions. The most apical portion was frozen in liquid nitrogen and stored at \(-80^\circ\)C until being used to assay caspase-3–like activity. The remaining portion was fixed in 10% buffered formalin for conventional histology, electron microscopy, and terminal deoxynucleotidyl dUTP nick end-labeling (TUNEL).

**Caspase-3–Like Activity Assay**

Caspase-3–like activity was measured by use of the ApoAlert Caspase-3 Fluorescent Assay Kit (Clontech) according to the manufacturer’s instructions. The substrate was DEVD-AFC. Approximately 150 \( \mu \)g of each cardiac homogenate was analyzed. The incubation time was 1 hour. Results were normalized to the exact amount of total protein assayed for each sample.

**Conventional Histology and TUNEL**

The formalin-fixed transverse ventricular slices were embedded in paraffin and cut into 4-\( \mu \)-m serial sections that were used for hematoxylin and eosin staining and TUNEL. TUNEL was performed with the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After TUNEL, all nuclei were counterstained with TOTO-3 iodide (1:200; Molecular Probes) and 1 \( \mu \)g/mL 4',6-diamidino-2-phenylindole (DAPI). To identify myocytes, actin was stained with phallolidin conjugated to tetramethyl rhodamine isothiocyanate (1:200; Sigma). For each specimen, the number of TUNEL-positive myocytes and the number of total myocytes were counted in 60 random high-power fields (x400). Approximately 3000 total myocytes were examined per section. The percentage of total myocytes that were TUNEL-positive (apoptotic index) was then calculated. This evaluation was performed by one person who was blinded to treatment group.

**Electron Microscopy**

Ventricular samples were cut into 1-mm cubes and fixed for 4 hours at 4\(^\circ\)C in 2.5% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4). They were postfixed in 1% buffered osmium tetroxide, dehydrated through graded ethanol, and embedded in epoxy resin. Thin sections (80 nm) were cut with a diamond knife, collected on 300-mesh copper grids, and double-stained with uranyl acetate and lead citrate before examination with an electron microscope (H-700, Hitachi).

**Statistical Analysis**

All values are presented as mean \( \pm \)SEM. Statistical significance was evaluated with Student’s \( t \) test for paired comparisons or ANOVA followed by Newman-Keuls test for analysis of multiple groups. Kaplan-Meier analysis was used for survival comparison between groups. Differences were considered statistically significant at a value of \( P < 0.05 \).

**Results**

**Caspase-3–Like Activity**

To determine whether treatment with IDN-1965 decreased effector caspase activity in the hearts of pregnant Gaq transgenic mice, caspase-3–like (DEVD-ase) activity was assessed at postpartum day 14 and found to be 86.5% lower \((P < 0.01)\) in mice treated with IDN-1965 than in those receiving vehicle (Figure 1). The suppression was complete, because the caspase-3–like activity in the hearts of IDN-1965–treated mice did not differ from that in the hearts of nonpregnant Gaq transgenic and wild-type mice. Thus, continuous subcutaneous delivery of this potent polycaspase inhibitor is sufficient to suppress effector caspase activity.

**Cardiac Myocyte Apoptosis**

To determine the effect of caspase inhibition on cardiac myocyte apoptosis, cell death was assessed both by TUNEL staining using light microscopy and analysis of cellular morphology using electron microscopy. Figure 2A shows a typical example of intense TUNEL staining of a myocyte...
nucleus from the heart of a postpartum day 14 vehicle-treated Gq transgenic mouse. Figure 2B illustrates the classic pattern of chromatin condensation against the inner surface of the nuclear membrane in an apoptotic cardiac myocyte from another vehicle-treated postpartum Gq transgenic mouse. Necrotic morphology, as defined by disruption of the plasma membrane, was rarely seen in any of the specimens. The magnitude of cardiac myocyte apoptosis in the 2 groups was quantified by use of TUNEL (Figure 2C). The percentage of TUNEL-positive cardiac myocytes was 1.9\pm0.3% in the vehicle-treated group, compared with 0.2\pm0.1% in the group treated with IDN-1965 (P<0.01). Therefore, treatment of pregnant Gq transgenics with IDN-1965 markedly decreased the frequency of cardiac myocyte apoptosis.

Echocardiography
To determine the effect on LV chamber size and contractile function of inhibiting cardiac myocyte apoptosis in pregnant Gq mice, echocardiography was performed at postpartum day 14 in vehicle- and IDN-1965–treated mice. Parallel studies were also performed in age-matched, nonpregnant, female wild-type and Gq mice. Before pregnancy, LV end-diastolic dimension (LVEDD) (Figure 3A) was increased in Gq mice compared with wild-type mice, consistent with the known baseline dilated cardiomyopathy in transgenics (wild-type, 2.3\pm0.1 mm; transgenics, 3.5\pm0.1 mm; P<0.01). LVEDD in postpartum transgenics was increased further to 4.7\pm0.1 mm (P<0.01 compared with nonpregnant transgenics). Treatment of pregnant transgenics with IDN-1965 decreased LVEDD 10.6% compared with vehicle treatment (vehicle, 4.7\pm0.1 mm; IDN-1965, 4.2\pm0.1 mm; P<0.01).

Abnormalities in fractional shortening (FS) (Figure 3B) paralleled those in LVEDD. Thus, FS was decreased in nonpregnant Gq mice compared with nonpregnant wild-type mice (wild-type, 75.9\pm1.4%; transgenic, 44.1\pm1.4%; P<0.01), consistent with the baseline cardiomyopathy in transgenics. FS in postpartum transgenics was decreased...
Discussion

This study tests whether cardiac myocyte apoptosis plays a causal role in the pathogenesis of heart failure. The model studied involves the overexpression of Gq in the myocardium, which results in a baseline dilated cardiomyopathy that becomes fulminant and lethal during pregnancy. The results demonstrate that 89% reduction in cardiac myocyte apoptosis...
by caspase inhibition ameliorates the peripartum cardiac dysfunction approximately halfway to baseline. Strikingly, however, the 30% mortality characteristic of this model was completely ablated.

Over the past decade, the cardiac apoptosis field has struggled with understanding the importance, or lack thereof, of the low frequencies of cardiac myocyte apoptosis, 0.08% to 0.25%,10-21 noted in human hearts with advanced failure. Does this cell death play a mechanistic role in the pathogenesis of dilated cardiomyopathy, or is it a parallel, unrelated event? A previous study from our laboratory addressed this issue directly by expressing a ligand-activatable procaspase-8 allele exclusively in the hearts of transgenic mice.37 These mice exhibited spontaneous apoptotic indices of 0.023%, levels that are 15-fold elevated over baseline but that are still 4- to 10-fold lower than those seen in human heart failure, and they developed a lethal diluted cardiomyopathy over 2 to 6 months. This phenotype was preventable by caspase inhibition. Although this previous study established for the first time that very low levels of cardiac myocyte apoptosis are sufficient to produce heart failure (and, in fact, were necessary in that system), it did not assess whether cardiac myocyte apoptosis plays a critical role in heart failure pathogenesis in models of more physiological relevance.

Hence, in the present study, we turned our attention to a genetic model that accurately replicates the transition of compensated LV hypertrophy to diluted cardiomyopathy.23 We chose this model for several reasons. First, hypertrophic signaling is generally thought to be involved in the pathogenesis of heart failure, and the myocardial overexpression of Gaq leads to activation of pathways downstream of multiple clinically relevant hypertrophic signals (eg, angiotensin II, endothelin-1, norepinephrine, etc).22 Second, the functional abnormalities and mortality resulting from the peripartum cardiomyopathy of Gaq mice are both reproducible and robust.9 Third, the defined 2- to 3-week time frame of the peripartum cardiomyopathy makes the model amenable for chronic administration of a caspase inhibitor. Our data show that reduction of cardiac myocyte apoptosis by caspase inhibition results in amelioration of cardiac dysfunction and complete suppression of mortality. This demonstrates that cardiac myocyte apoptosis indeed plays a critical role in the pathogenesis of heart failure in this model. Having shown the importance of cardiac myocyte apoptosis for the development of heart failure in this clinically relevant genetic model, it will be important to assess whether cardiac myocyte apoptosis is also important in surgical models of hemodynamic overload, including those caused by transverse aortic constriction and remote myocardial infarction, in which rates of cardiac myocyte apoptosis are substantially lower.

Elegant studies have recently elucidated a mechanism by which Gaq overexpression elicits apoptosis: the transcriptional upregulation of the BH3-only proapoptotic Bcl-2 family member Nix/Bnip3L, which localizes at the mitochondria and triggers cytochrome c release.36 The relevance of this mechanism is underscored by the fact that a truncated, dominant negative Nix isoform ameliorates the Gaq peripartum cardiomyopathy. Given the importance of the mitochondrial death pathway in the Gaq transgenics, a key question concerns the mechanisms by which caspase inhibitors are blocking apoptosis and rescuing the peripartum cardiomyopathy phenotype. In light of the significant mitochondrial damage that results from Gaq overexpression in cardiac myocytes,35 it would be surprising if mere inhibition of downstream (postmitochondrial) effector caspases 3, 6, and 7 were adequate to bring about rescue. In most biological systems, however, the mitochondrial release of cytochrome c is independent of caspase activation, and this has been shown to be the case for cardiac myocytes30 and specifically in response to Gaq overexpression.35 A possible resolution of this conundrum is that caspase inhibition may be blunting mitochondrial injury through another means. One possibility is by inhibiting the known caspase-dependent38 mitochondrial release of Smac (second mitochondrial activator of cytochrome c)/DIABLO (direct IAP binding protein with low pI),39,40 which promotes apoptosis after its release from mitochondria by binding to IAPs (inhibitor of apoptosis proteins), resulting in the competitive displacement and disinhibition of the caspases.41 Future studies are needed to assess whether this mechanism operates in this and other models in which caspase inhibition seems to limit mitochondrial damage.

The data indicate that caspase inhibition resulted in an 89% reduction in cardiac myocyte apoptosis. This reduction in cell death is the most likely mechanism for the rescue of the Gaq peripartum cardiomyopathy. Another non–mutually exclusive possibility, however, is that caspase inhibition may have resulted in improvements in cardiac function apart from its inhibition of cell death. In support of this concept, several articles have provided preliminary evidence that sarcomeric components may be caspase substrates.42-44 Further studies in which noncleavable mutants of these putative caspase targets are introduced into cultured cardiac myocytes will be needed to carefully sort out the functional significance of these cleavage events.

Whatever its precise mechanisms of action, the fact that caspase inhibitors so effectively rescued the Gaq cardiomyopathy raises the possibility that drugs in this class may ultimately be useful in treating human heart failure. The next steps in testing this notion are to evaluate the generality of these inhibitors in different rodent and selected large-animal models of
heart failure. Another issue that will need to be evaluated is the long-term safety of these agents, especially with respect to carcinogenesis. Dilated cardiomyopathy is a lethal syndrome, however, and the use of antiapoptotic therapies may ultimately require a risk-benefit analysis similar to that involved in the decision to use anthracycline cancer chemotherapy, which can cause dilated cardiomyopathy.

In summary, this study shows for the first time that reduction of cardiac myocyte apoptosis by caspase inhibition improves cardiac function and extinguishes mortality in the Gq peripartum cardiomyopathy model. These data strongly support the conclusion that myocyte apoptosis is a critical component of heart failure in this model.

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References
11. Hayakawa et al Causal Role of Myocyte Apoptosis in Heart Failure 3041
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