Nix-Mediated Apoptosis Links Myocardial Fibrosis, Cardiac Remodeling, and Hypertrophy Decompensation

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Background—Pathological cardiac hypertrophy inevitably remodels, leading to functional decompensation. Although modulation of apoptosis-regulating genes occurs in cardiac hypertrophy, a causal role for programmed cardiomyocyte death in left ventricular (LV) remodeling has not been established.

Methods and Results—We targeted the gene for proapoptotic Nix, which is transcriptionally upregulated in pressure overload and Gq-dependent hypertrophies, in the mouse germ line or specifically in cardiomyocytes (knockout [KO]) and conditionally overexpressed it in the heart (transgenic [TG]). Conditional forced Nix expression acted synergistically with the prohypertrophic Gq transgene to increase cardiomyocyte apoptosis (0.8±0.1% in GqTG versus 7.8±0.6% in GqTG+NixTG; $P<0.001$), causing lethal cardiomyopathy with LV dilation and depressed systolic function (percent fractional shortening, 39±4 versus 23±4; $P=0.042$). In the reciprocal experiment, germ-line *Nix* ablation significantly reduced cardiomyocyte apoptosis (4.8±0.2% in GqTG+*Nix*KO versus 8.4±0.5% in GqTG; $P=0.001$), which improved percent fractional shortening (43±3% versus 27±3%; $P=0.017$), attenuated LV remodeling, and largely prevented lethality in the Gq peripartum model of apoptotic cardiomyopathy. Cardiac-specific (Nkx2.5-Cre) *Nix* KO mice subjected to transverse aortic constriction developed significantly less LV dilation by echocardiography and magnetic resonance imaging, maintained concentric remodeling, and exhibited preserved LV ejection fraction (61±2% in transverse aortic constriction cardiac *Nix* KO versus 36±6% in transverse aortic constriction wild-type mice; $P=0.003$) at 9 weeks, with reduced cardiomyocyte apoptosis at day 4 (1.70±0.21% versus 2.73±0.35%; $P=0.032$).

Conclusions—Nix-induced cardiomyocyte apoptosis is a major determinant of adverse remodeling in pathological hypertrophies, a finding that suggests therapeutic value for apoptosis inhibition to prevent cardiomyopathic decompensation. (***Circulation*. 2008;117:396-404.**)

Key Words: apoptosis ■ cardiomyopathy ■ hypertrophy

Cardiac hypertrophy is an independent risk factor for death largely because chronically hypertrophied hearts remodel and dilate, progressing from a stable compensation to dilated cardiomyopathy.[1] A better understanding of the mechanisms for functional decompensation of cardiac hypertrophy in response to hemodynamic overload is essential to develop effective preventative measures and therapeutics. A number of candidate pathological events have been identified, including bioenergetically unfavorable changes in contractile protein isoforms,[2] the metabolically adverse transition from fatty acid to glucose utilization,[3,4] degradation of the cardiac matrix and resulting myocyte slippage,[5] and a relative decrease in myocardial vascularization resulting in oxidative stress.[6] Each of these factors becomes an additional physiological stressor for hemodynamically overloaded cardiomyocytes and in combination with the primary stimulus can ultimately overwhelm protective cell survival pathways[5] and result in apoptotic cardiomyocyte dropout with replacement fibrosis. Apoptotic loss of myocardium itself can increase hemodynamic stress through ventricular dilation and wall thinning and is therefore hypothesized to play an important role in the downward functional spiral that ultimately leads to overt heart failure.[5,7] Although cardiomyocyte apoptosis is commonly observed in pressure overload hypertrophy[7–10] and causes myocardial disease in experimental models wherein it was artificially induced,[11–15] the degree to which it contributes to ventricular remodeling in naturally occurring hypertrophy decompensation remains unclear. It is also not known whether cardiac myocytes that are programmed to die in pressure overload hypertrophy may nevertheless die a necrotic death if apoptosis is prevented, in which case inhibiting apoptosis would likely prove ineffective in preventing decompensation.

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One approach to testing the hypothesis that apoptosis is a critical pathophysiological nodal point for multiple factors that induce hypertrophy decompensation, as well as for testing the therapeutic efficacy of apoptosis prevention to interrupt the feed-forward cycle of functional deterioration of pressure overload hypertrophy, is pharmacological caspase inhibition.[16] This approach is limited, however, by potential toxicity and lack of absolute specificity of these compounds, and it has not been reported in pressure overload hypertrophy. We considered that a more selective tactic of identifying proapoptotic factor(s) responsible for apoptosis after cardiac pressure overloading and individually manipulating them in the heart could better validate the hypothesis. Previously, we used DNA microarray analysis to identify Nix as a candidate hypertrophy-stimulated proapoptotic factor.[17] Nix is a nearly ubiquitous member of the Bcl2 family of mitochondrial-localized proteins that is expressed at very low levels in normal hearts but was strikingly upregulated in cardiac-specific Gq-overexpressing mice, a genetic model that recapitulates the molecular pathways and essential features of pressure overload hypertrophy.[18,19] Nix provokes apoptosis in transfected cells and acts synergistically with normal maturational growth to cause apoptotic heart failure in neonatal mice, but its overexpression alone was not sufficient to cause apoptotic cardiac decompensation in normal adult hearts.[13,15] It is not known whether Nix is essential for apoptosis that occurs in Gq-mediated or pressure overload hypertrophy and therefore represents a specific, targetable apoptotic effector of hypertrophy decompensation. To test this, we used cardiac-specific conditional Nix gain- and loss-of-function mouse models in combination with genetic and physiological stress. Our results support a critical role for Nix in remodeling of hemodynamically overloaded hearts and more clearly define the consequences of cardiomyocyte apoptosis in functional decompensation after pressure overload hypertrophy.

Methods

Generation and Characterization of Genetically Modeled Mice

Mice with cardiac-specific Gq overexpression or inducible, attenuated expression of Nix were described previously[13,18] and were interbred. Gq transgenic mice were mated with *Nix* null mice[20] for peripartum cardiomyopathy studies.

To produce cardiac *Nix* del animals, mice homozygous for floxed *Nix* alleles ($Nix^{f/f}$)[20] were mated with Nkx-Cre knockin mice.[21] $Nix^{f/f}$ mice were used as wild-type (WT) controls. Mice were housed and studied according to procedures approved by the University of Cincinnati Institutional Animal Care and Use Committee. Two-dimensional directed M-mode echocardiography, histopathology, and terminal deoxynucleotidyl transferase–mediated dUTP biotin nick end labeling (TUNEL) studies were performed as described previously.[13]

Expression Analysis With Quantitative Polymerase Chain Reaction

One microgram of total RNA purified from snap-frozen mouse hearts with the use of Trizol (Invitrogen, Carlsbad, Calif) was reverse-transcribed with the use of oligo(dT). Amplicons spanning exons 2 to 4 of Nix (Bnip3L, GenBank NM_009761, nucleotides 298 to 414) were detected with the use of SYBR Green I during 35 cycles of quantitative polymerase chain reaction (95°C for 15 seconds, 60°C for 1 minute) with the use of 5′-AAGAGGCAGTTCGCACTGTGACA-3′ (forward) and 5′-TCTACAACTTCTTCTTCTGACTGAGAGCTG-3′ (reverse) primers. TaqMan assays for atrial natriuretic factor, SERCA2a, α-skeletal actin, and GAPDH were purchased from Applied Biosystems (Foster City, Calif).

Studies With Pressure Overload Modeling

Twelve-week-old mice underwent acute pressure overloading by surgical transverse aortic constriction (TAC).[19] Periprocedural mortality was ≈25% in both groups. Cardiac magnetic resonance imaging studies were performed as described.[22] Terminal invasive studies for assessment of transcoarctation gradient and left ventricular (LV) hemodynamics were performed as described previously.[19]

Myocardial Histomorphometric Analysis

Myocyte cross-sectional area was determined with the use of FITC-tagged wheat germ agglutinin labeling.[23] TUNEL staining used the DeadEnd fluorometric TUNEL system (Promega, Wis) counterstained with α-sarcomeric actin (Invitrogen) and DAPI (Vector Laboratories, Burlingame, Calif) and imaged with the use of a UV transparent ×100 oil immersion objective. Only TUNEL-positive nuclei within sarcomeric actin–stained cardiomyocytes were counted. Cleaved caspase-3 and poly(ADP-ribose) polymerase (PARP) were analyzed as described.[24] Masson's trichrome–stained myocardial sections were imaged (×200), and collagen area was calculated as percentage of total LV myocardial area with the use of NIH Image software.

Statistical Analysis

Results are mean±SEM. Experimental groups were compared with the use of Student *t* test for comparison between 2 groups, 1-way ANOVA for comparing multiple groups, or 2-way repeated-measures ANOVA for time-dependent changes between various groups, followed by the Tukey post hoc test. Nonparametric testing was employed when data were not normally distributed. The Dunn post hoc test was employed after ANOVA on ranks. The log-rank test was employed for survival analyses. $P<0.05$ was considered significant.

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

Results

Nix Synergizes With Gq-Mediated Hypertrophy to Cause Lethal Apoptotic Heart Failure

Nix gene expression increases in human and experimental pressure overload hypertrophy.[15,17,24] We previously found that increased expression of Nix alone did not cause functionally significant cardiomyocyte apoptosis in normal adult mice but that Nix caused progressive apoptotic cardiac dilation in neonatal mice[13] and that proapoptotic effects of Nix were inversely proportional to the rate of maturational cardiomyocyte growth. Here, to define possible synergies between Nix and pathological cardiomyocyte growth,[25] we examined the consequences of conditional forced Nix expression in mice with Gq-mediated hypertrophy. Overexpression of Gq is a cardiac myocyte–specific genetic stimulus for pathological hypertrophy that recapitulates many characteristics of compensated pressure overload hypertrophy.[18,19]

As previously observed,[13] cardiac-specific overexpression of Nix beginning in the neonatal period (Figure 1A) increased cardiomyocyte apoptosis to levels (≈4%; Figure 1D) that, over time, significantly diminished echocardiographic LV fractional shortening (percent fractional shortening [%FS]) (Figure 1C, black bars). Survival to adulthood was not affected (Figure 1B), nor was LV remodeling induced, measured as the ratio of

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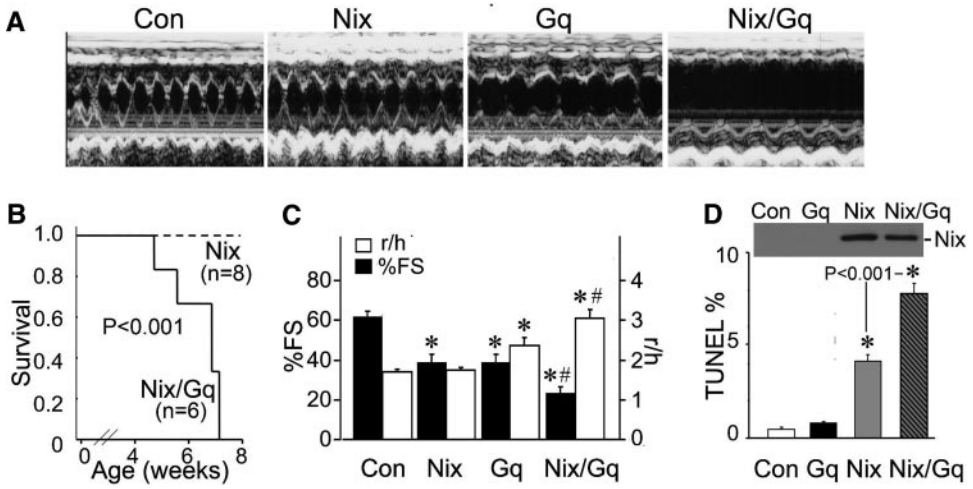

Figure 1. Apoptotic synergy between cardiac-expressed Nix and Gq in neonatal mice. A, Representative LV M-mode echocardiograms in short-axis view at 6 weeks of age. B, Survival curves (probability value by log-rank test). C, Echocardiographic LV fractional shortening (black, %FS) and remodeling (LV radius/wall thickness, white, r/h; n=4 to 8 per group). D, Representative Western blot for Nix (inset) and apoptotic indices by TUNEL analysis (n=3 per group). *$P<0.05$ vs control, #$P<0.05$ for Nix/Gq vs Gq by post hoc test after 1-way ANOVA.

ventricular radius to wall thickness (r/h) (Figure 1C, white bars). Gq transgenic mice exhibited characteristic functional impairment (decreased %FS) and mild ventricular remodeling (Figure 1A and 1C), without cardiomyocyte apoptosis (Figure 1D) or mortality (not shown).[18,19] Strikingly, although Nix expression was similar in Nix and Nix/Gq compound transgenic hearts (Figure 1D, inset), combined postnatal cardiac overexpression of Nix and Gq proved rapidly lethal (Figure 1B). At 6 weeks, cardiomyocyte apoptosis was doubled in Nix/Gq mice compared with Nix alone (Figure 1D), with ventricular remodeling (Figure 1A and Figure 1C, white bars) and dramatically depressed %FS (Figure 1A and 1C, black bars). These results demonstrate that Gq-mediated pathological hypertrophy exacerbates apoptosis caused by Nix overexpression, providing additional evidence for synergy between cardiac growth and death pathways.[25]

Nix Gene Ablation Prevents Gq-Mediated Apoptotic Peripartum Cardiomyopathy

Nix is upregulated in Gq-mediated cardiac hypertrophy,[15,17] and the aforementioned results show that Gq-mediated hypertrophy contributes to apoptosis caused by Nix expression. A unique manifestation of apoptotic myocardial disease in the Gq mouse

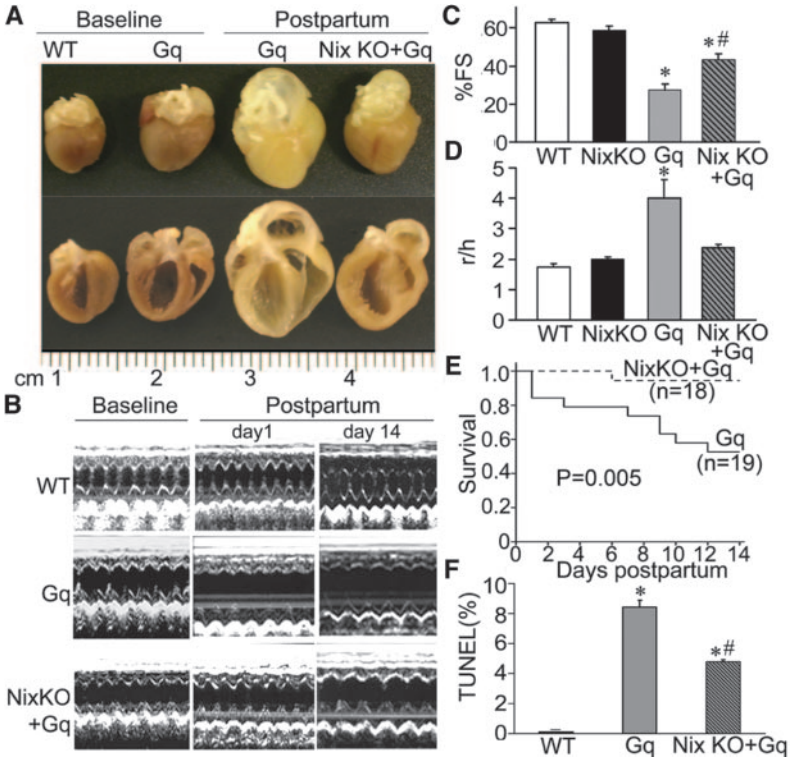

Figure 2. Nix gene ablation diminishes apoptosis in Gq-mediated peripartum cardiomyopathy, improving function and minimizing death. A, Representative nonpregnant (baseline) and postpartum day 14 hearts. B, Representative LV M-mode echocardiograms. C, Echocardiographic LV fractional shortening (%FS). D, Remodeling (LV radius/wall thickness, r/h; n=4 to 6 per group). E, Kaplan-Meier survival curves for peripartum Gq expressors, with and without Nix (probability value by log-rank test). F, Apoptotic indices at 1 day postpartum (n=4 per group). *$P<0.05$ vs WT, #$P<0.05$ for Nix KO+Gq vs Gq by post hoc test after 1-way ANOVA.

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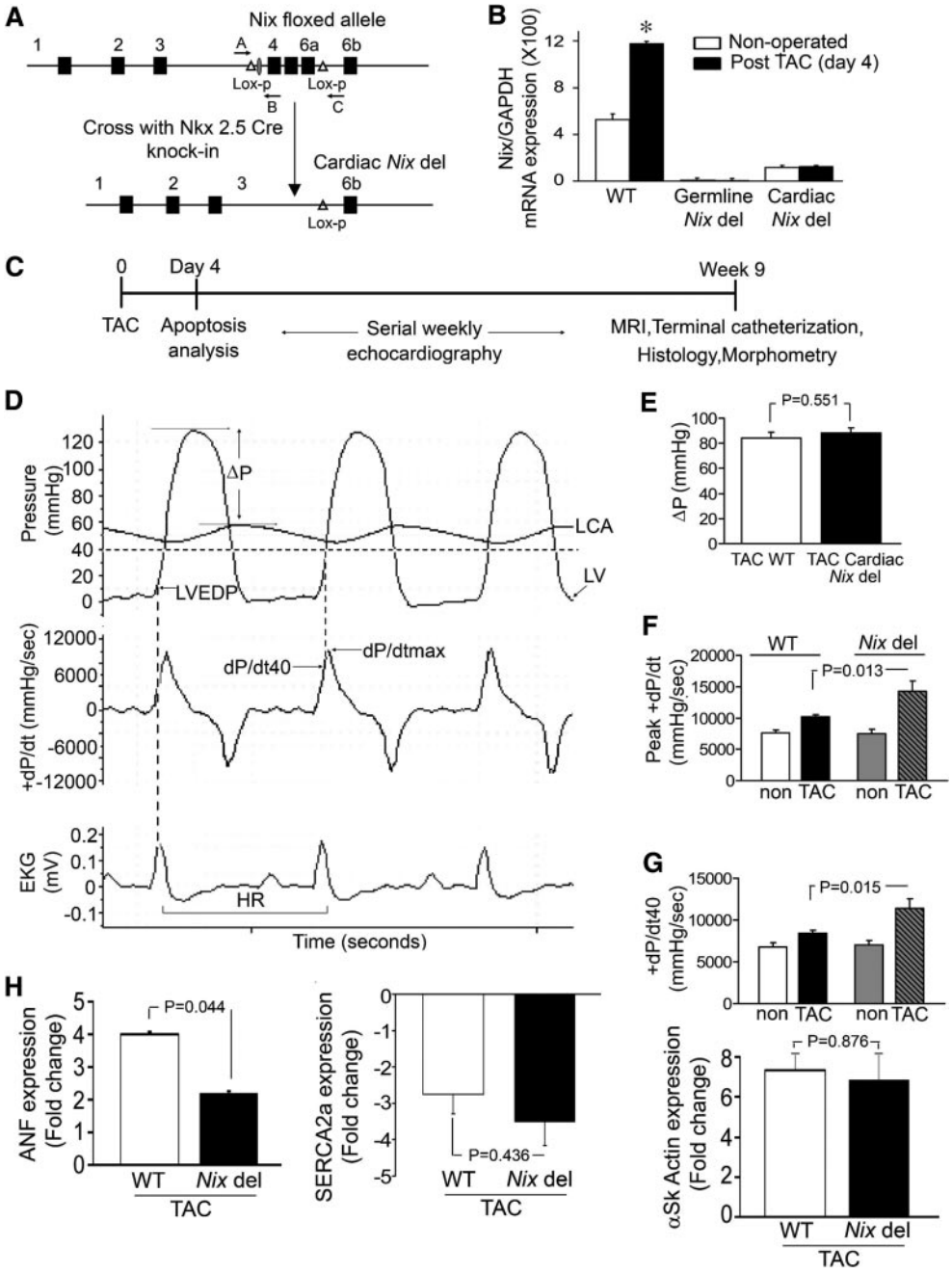

Figure 3. Cardiac-specific Nix ablation prevents functional decompensation after acute TAC. A, Cardiac-specific *Nix* ablation strategy. B, Quantitative polymerase chain reaction of Nix mRNA expression after TAC (n=3 per group). *$P<0.05$ vs nonoperated by *t* test. C, Schematic of TAC modeling studies. D, Representative invasively determined hemodynamic tracings after TAC. ΔP indicates transcoarctation gradient; LVEDP, LV end-diastolic pressure; and HR, heart rate. E, Transcoarctation gradient in cardiac *Nix* del mice (n=11) and controls (n=13). Probability value reported is by *t* test. MRI indicates magnetic resonance imaging. Peak positive dP/dt (F) and dP/dt at 40 mm Hg (G) LV pressure in cardiac *Nix* del mice and *Nix* floxed controls subjected to TAC (n=4 per nonoperated group; n=11 to 13 per TAC group; probability values reported are by *t* test for comparison of TAC groups). Nonoperated mice are shown for comparison. H, Change in atrial natriuretic factor (ANF), SERCA2a, and α-skeletal (αSk) actin mRNA levels 4 days after TAC in WT (white bars) and cardiac *Nix* del (black bars).

is lethal peripartal heart failure.[16,23] To demonstrate that Nix-mediated apoptosis contributes to peripartal heart failure in the Gq mouse, we performed a genetic rescue experiment by crossing Gq transgenic mice to mice lacking a functional *Nix* gene (*Nix* null). Germ-line *Nix* null mice exhibit diminished apoptosis during normal erythroblast maturation[20] but have no detectable cardiac abnormalities (A.D. and G.W.D., unpublished data, 2007, and Figure 2C and 2D). Crossing α-myosin heavy chain–directed Gq transgenic mice with *Nix* null mice produces a model in which Gq expression is cardiomyocyte specific and *Nix* ablation is systemic. Thus, cardiac phenotypes reflect the cardiac specificity of the hypertrophy stimulus.

No cardiac effects of parturition or of the peripartum state were observed in *Nix* null mice (not shown). As observed previously,[16,23] Gq mice developed peripartal cardiac enlargement and chamber dilation (Figure 2A and

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Table 1. Morphometric and Hemodynamic Parameters of Cardiac *Nix* del Mice 9 Weeks After TAC

	Nonoperated WT (n=6)	TAC WT (n=13)	Percent Change	Nonoperated Cardiac *Nix* del (n=6)	TAC Cardiac *Nix* del (n=11)	Percent Change	*P*
Heart/body weight, mg/g	4.9±0.1	8.8±0.9*	79±16	5.3±0.2	9.1±0.5*	71±10	0.954
Peak LV pressure, mm Hg	99±5	161±5*	62±5	98±7	171±6*	75±6	0.093
Peak positive dP/dt, mm Hg/s	7596±441	10 189±368*	34±5	7507±627	14 304±1571*	91±21	0.006
Peak negative dP/dt, mm Hg/s	−8070±576	−12 090±368*	50±5	−7740±589	−15 688±1756*	103±23	0.056
dP/dt40, mm Hg/s	6742±569	8432±311*	25±4	6990±567	11 368±1154*	63±17	0.049
LV end-diastolic pressure, mm Hg	3±2	10±1*	243±45	4±2	10±2	160±60	0.301

All data are shown as mean±SEM. *P* values are for comparison of percent change in TAC group between WT and cardiac *Nix* del by Student *t* test.
*$P<0.05$ vs respective nonoperated group by Student *t* test.

2B) with striking LV remodeling (increase in r/h; Figure 2D) and a >50% decline in LV fractional shortening (Figure 2B and 2C). Mortality from heart failure in peripartum Gq mice was ≈50% (Figure 2E), and the rate of cardiomyocyte apoptosis (TUNEL positivity) on the first postpartum day was ≈8% (Figure 2F). By comparison, syngeneic Gq mice lacking a functional *Nix* gene had reduced peripartal cardiac enlargement and LV dilatation (Nix knockout [KO]+Gq; Figure 2A and 2B), without remodeling but with significantly improved ejection performance and decreased mortality and cardiomyocyte apoptosis (Figure 2C through 2F). These data establish the importance of Nix-mediated apoptosis in the development of, and mortality that results from, apoptotic cardiomyopathy in peripartum Gq overexpressing mice.

Cardiomyocyte-Specific Ablation of Nix Prevents Decompensation of Pressure Overload Hypertrophy

Taken together, the aforementioned results show that Nix is necessary and sufficient for apoptotic decompensation of Gq-mediated hypertrophy. To determine whether Nix plays an analogous role in pressure overload hypertrophy (which is also Gq dependent[26,27]) in a manner that would not be confounded by extracardiac effects of germ-line Nix ablation,[20] we ablated the *Nix* gene specifically in cardiomyocytes using Nkx-2.5–driven Cre[21] (Figure 3A). Cardiomyocyte-specific *Nix* KO mice (cardiac *Nix* del) were viable and exhibited no cardiac abnormalities by gross morphometry or echocardiography (Table 1 and data not shown). Compared with WT control mice, myocardial Nix mRNA levels measured by real-time quantitative polymerase chain reaction were decreased by 78% at baseline in cardiac *Nix* del mice and showed no change 4 days after imposition of an ≈84 mm Hg transaortic gradient by surgical TAC compared with doubling of Nix mRNA after TAC in WT mice (Figure 3B). Because Nix mRNA was not detectable with the use of this assay in hearts from germ-line *Nix* null mice (Figure 3B), Nix mRNA at baseline and after TAC in cardiac *Nix* del hearts likely represents constitutive Nix gene expression in nonmyocyte myocardial cells.[28–32]

Cardiac *Nix* del and WT mice underwent surgical TAC,[19] producing gradients of 88±4 and 84±4 mm Hg, respectively (*P*=NS; Figure 3D [top] and 3E). The time courses of hypertrophy development (LV mass), functional decompensation (%FS), and LV remodeling (LV end-diastolic dimension and r/h) were determined by serial echocardiography, followed by terminal invasive hemodynamic studies, cardiac morphometrics, and histological examinations of the myocardium (Figure 3C). Gravimetric cardiac hypertrophy measured 9 weeks after TAC, hypertrophy-associated changes in α-skeletal actin and sarcoplasmic reticulum ATPase (SERCA2a) gene expression measured 4 days after TAC, and the rate of LV hypertrophy development determined by weekly echocardiography (Table 1 and Figures 3H and 4A) were identical in cardiac *Nix* del mice and WT controls, showing that absence of Nix does not affect the hypertrophic response to pressure overload. In contrast, terminal invasive functional assessments of cardiac *Nix* del mice revealed enhanced myocardial contractile function late after pressure overloading measured as peak positive LV dP/dt or dP/dt at 40 mm Hg LV systolic pressure (Figure 3D, 3F, and 3G and Table 1). Echocardiography confirmed enhanced systolic performance (Figure 4D to 4F) and further demonstrated that the differences between TAC WT and cardiac *Nix* del mice in LV

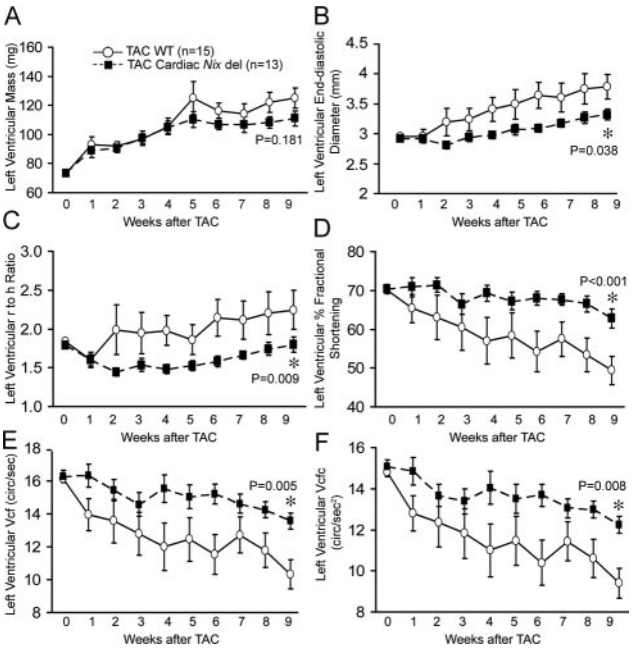

Figure 4. Cardiac-specific Nix ablation prevents structural remodeling and functional decompensation after TAC. Time-dependent echocardiographic outcomes after TAC in control (Nix floxed, white circles, n=15) and cardiac *Nix* del (black squares, n=13) mice. A, LV mass. B, LV end-diastolic dimension. C, Ratio of LV radius to wall thickness (r/h). D, LV %FS. E, Vcf indicates velocity of circumferential shortening. F, Vcfc indicates Vcf corrected for heart rate. Probability values by post hoc test vs WT TAC after 2-way repeated-measures ANOVA.

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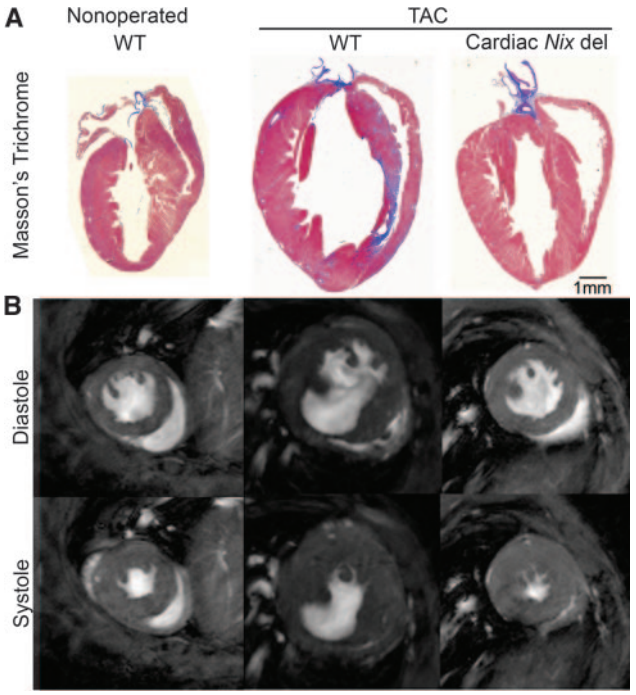

Figure 5. Cardiac-specific Nix ablation abrogates LV remodeling after TAC. A, Representative Masson's trichrome–stained coronal sections. B, Representative magnetic resonance images at 9 weeks after TAC.

diastolic chamber size (Figure 4B), remodeling (r/h; Figure 4C), and systolic performance (Figure 4D through 4F) were observed by 2 weeks after TAC, with the trend lines remaining roughly parallel thereafter. These findings, together with a significantly smaller increase in failure-associated atrial natriuretic factor mRNA levels (Figure 3H), suggest that the benefits of cardiac myocyte *Nix* ablation on LV remodeling and ejection performance in pressure overloaded hearts largely accrue early after TAC, when apoptosis is most active.

To better assess the consequences of cardiomyocyte-specific *Nix* ablation on ventricular remodeling and contractile function after pressure overloading, a cohort of surgically pressure overloaded mice underwent magnetic resonance imaging.[33,34] Compared with nonoperated controls, magnetic resonance imaging of WT mice 9 weeks after TAC showed increased LV mass and end-diastolic volume, with reduced sphericity index (increased sphericity), and an ≈50% decline in volumetric ejection fraction (Figure 5A and 5B and Table 2), representing typical LV remodeling and functional deterioration after acute pressure overloading.[35] In contrast, and consistent with the echocardiographic and invasive hemodynamic findings, pressure overloaded cardiac *Nix* del mice showed no significant LV dilation and had enhanced LV ejection fraction and a normal LV sphericity index, despite comparable hypertrophy (Figure 5A and 5B and Table 2). These results reinforce the findings of echocardiographic and terminal studies that *Nix* ablation prevents LV remodeling and minimizes LV functional declines in chronically pressure overloaded hearts.

Table 2. Magnetic Resonance Studies of LV Structure and Function in Cardiac *Nix* del Mice 9 Weeks After TAC

	Nonoperated (n=4)	TAC WT (n=7)	TAC Cardiac *Nix* del (n=5)
End-diastolic volume, μL	43±4	93±9*	51±4†
End-systolic volume, μL	13±1	62±10*	20±2
Ejection fraction, %	71±2	36±5*	61±2
Mass, mg	57±5	142±14*	114±16*
Sphericity index	1.96±0.05	1.56±0.06*	1.84±0.05†

All data are shown as mean±SEM. All groups were compared by 1-way ANOVA.

*$P<0.05$ vs nonoperated WT.

†TAC cardiac *Nix* del vs TAC WT by post hoc test (Tukey).

Cardiomyocyte-Specific Ablation of Nix Diminishes Myocardial Apoptosis in Response to Pressure Overload

Taken together, the aforementioned studies demonstrate that cardiac myocyte–specific *Nix* ablation largely prevents remodeling and functional deterioration in Gq-mediated and pressure overload hypertrophies. In the Gq model, the benefits of *Nix* ablation and the deleterious effects of Nix overexpression were associated with reciprocal effects on cardiac myocyte apoptosis. To interrogate the effects of Nix ablation on cardiac myocytes in pressure overload hypertrophy, studies of cardiac myocyte hypertrophy, apoptosis, and replacement fibrosis were performed. Cardiac myocyte cross-sectional area, which is increased in pressure overload hypertrophy,[36] showed similar ≈35% increases in WT and cardiac *Nix* del mice 9 weeks after TAC (Figure 6A), indicating that *Nix* ablation does not affect cellular hypertrophy.

Apoptosis studies in control mice 4 days after TAC showed an ≈5-fold increase in the number of TUNEL-positive cardiomyocytes compared with baseline (Figure 6B), with increased caspase 3 and PARP cleavage (Figure 6C). By comparison, cardiac *Nix* del hearts after TAC had less TUNEL positivity (Figure 6B) and caspase 3 and PARP cleavage (Figure 6C). Late fibrotic replacement of dead cardiac myocytes[37] was also reduced by approximately half in cardiac *Nix* del subjected to TAC compared with identically treated controls (Figure 6D). Because provocation of mitochondrial/intrinsic pathway apoptosis is the only known cellular function of Nix,[20] and Nix is specifically upregulated in pressure overload hypertrophy,[15,24] these results are consistent with the notion that the geometric and functional benefits afforded by cardiomyocyte *Nix* ablation after pressure overload are a consequence of reduced myocardial apoptotic cell loss.

Discussion

The present studies mechanistically link cardiomyocyte apoptosis with ventricular remodeling and functional deterioration during the transition from compensated pressure overload hypertrophy to decompensated heart failure. They further identify induction of *Nix* gene expression as a critical molecular event causing apoptotic cardiac myocyte dropout in pressure overload and Gq-mediated hypertrophy. The efficacy of cardiomyocyte salvage by apoptosis inhibition under conditions when no relief is provided from the primary inciting stimulus demonstrates that

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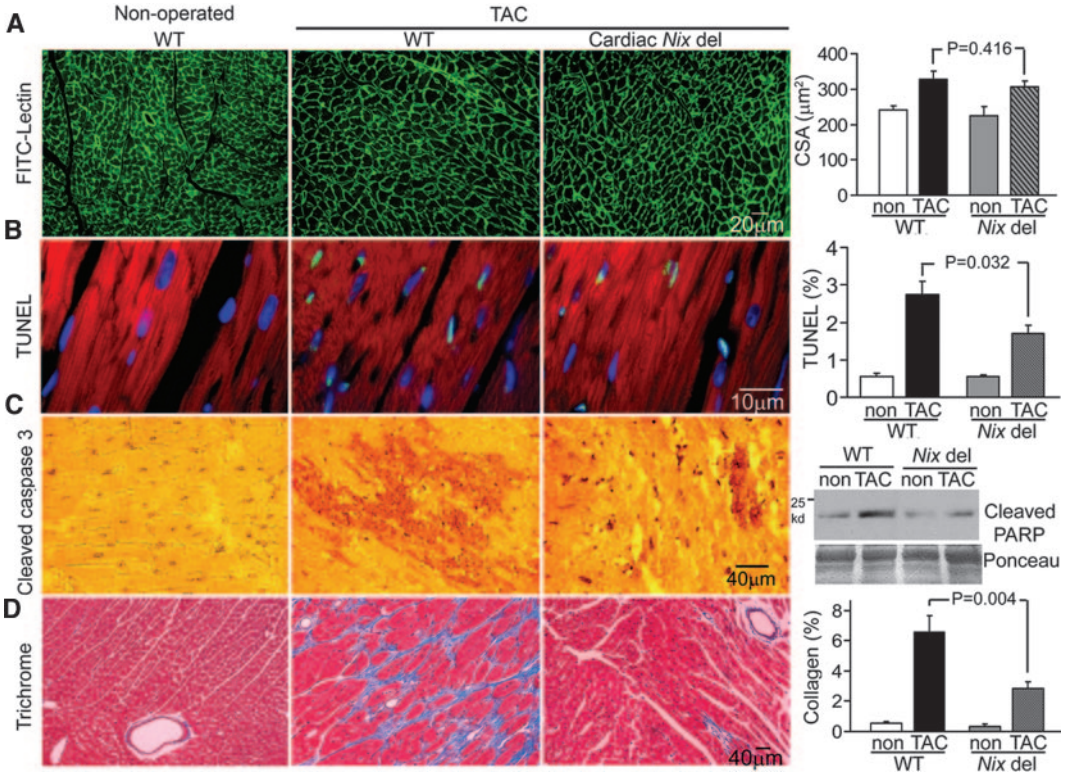

Figure 6. Cardiac-specific Nix ablation prevents cardiomyocyte apoptosis and myocardial fibrosis after TAC. A, Representative myocardial sections show cardiomyocyte cross-sectional area (FITC-tagged wheat germ agglutinin labeling, ×200). B, TUNEL positivity (×1000). C, Cleaved caspase 3 (brown staining); left panel (×400) and immunoblot (right) show cleaved PARP (top) with Ponceau red loading control (bottom). D, Myocardial fibrosis (Masson's trichrome, ×200) 9 weeks after TAC in cardiac *Nix* del mice and *Nix* floxed controls. Group data are in bar graphs to the right (n=4 per nonoperated group and n=6 to 12 per TAC group). Probability values by *t* test, TAC WT vs TAC *Nix* del. Nonoperated mice are shown for comparison.

preventing apoptotic cardiomyocyte death does not simply commit the cell to a necrotic one.

We selected Nix as the candidate effector of apoptosis for our studies of hypertrophy decompensation because Nix transcripts are specifically upregulated in pathological hypertrophy.[15,24] Nix protein rapidly localizes to mitochondrial outer membranes via a carboxyl-terminal hydrophobic localization domain, without which Nix lacks apoptotic activity.[15,38] Nix protein also undergoes rapid proteolytic degradation,[38,39] revealing that apoptosis induced by it is tightly regulated by both rapid "start" and "stop" functions. At the mitochondria, Nix promotes the release of cytochrome *c* by supporting permeabilization of the mitochondrial outer membrane,[20] almost certainly by stimulating oligomerization of the multidomain pore-forming Bcl-2 proteins Bax and Bak.[40] Addition of recombinant Nix to isolated mitochondria does not open permeability transition pores,[20] although this occurs in intact cells undergoing Gq-mediated apoptosis.[23,41,42] Thus, available evidence indicates that Nix is a hypertrophy-inducible apical regulator of apoptosis mediated strictly via the intrinsic, mitochondrial pathway.[43]

It is notable that *Nix* ablation in cardiac myocytes and the germ line did not affect the cardiac response to 2 hypertrophic stimuli. Thus, the functional and structural benefits of *Nix* ablation are independent of the magnitude of the hypertrophic response. This observation is not fully consistent with the proposition that "pathological hypertrophy" is intrinsically harmful, or at least fully dispensable, in pressure overload[44–46] and suggests that hypertrophy can be tolerated if its deleterious aspects are neutralized. This notion is also consistent with recent studies demonstrating that microischemia due to inadequate angiogenesis contributes to hypertrophy decompensation and that enhancing angiogenesis can prevent this.[6,47,48] It is interesting to speculate that microischemia in cardiac hypertrophy may actually contribute to hypertrophic *Nix* gene induction because hypoxia has been shown to increase Nix transcripts in cultured tumor cell lines,[49,50] and in the present studies Nix overexpression alone was not sufficient to cause apoptotic heart failure, whereas the same level of Nix expression in the context of Gq-mediated hypertrophy proved lethal.

Why was hypertrophy-associated apoptosis incompletely suppressed by *Nix* ablation? First, Nix may not be the only mitochondrial apoptotic factor induced in cardiac hypertrophy. We and others have shown that closely related Bnip3 is strongly induced in cardiomyocyte ischemia.[24,51,52] Bnip3 and Nix have nearly identical apoptotic effects in cells[38] and may interact cooperatively in the heart (G.W.D. and A.D., unpublished data). Recent evidence that failure of angiogenesis contributes to functional hypertrophy decompensation[6,47,53] suggests that cardiomyocyte ischemia may be unavoidable in pressure overload hypertrophy. If so, coinduction of hypertrophic and ischemic apoptotic factors may be the rule, and elimination of apoptosis will not be accomplished by targeting a single gene or gene product.

The second likelihood is that extrinsic, death receptor apoptosis pathways contribute to apoptosis after pressure

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overload. Certainly, loss of the interleukin-6/gp130 cytokine receptor and its cardiomyocyte survival function leads to dramatic apoptotic decompensation after pressure overloading.[12] The proinflammatory/proapoptotic cytokine tumor necrosis factor-α also contributes to decompensation of pressure overload hypertrophy.[54] However, although cross talk occurs between mitochondrial and death receptor apoptosis pathways,[55] Nix is not involved. Thus, it is likely that Nix modulates only apoptosis transduced via the intrinsic pathway in decompensating pressure overload hypertrophy but that death receptor pathways can also contribute.

In conclusion, the present results support the proposition that programmed cardiomyocyte death is at a junction between multiple mechanical and molecular factors that contribute to heart failure, and they demonstrate that moderating cardiomyocyte apoptosis can interrupt the cycle of physiological stress leading to myocyte dropout that, in turn, exacerbates the physiological stress. Retention of cardiac myocytes that were programmed to die is a form of myocardial salvage that helps to maintain normal chamber wall thickness and dimension and therefore preserves cardiac ejection performance and hemodynamic homeostasis.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Left ventricular hypertrophy after hemodynamic overload tends inexorably to functionally decompensate through the process of ventricular remodeling. Ventricular dilation and diminished ejection performance in remodeled ventricles have been associated with histological changes reflecting "dropout" of cardiac myocytes and their replacement with fibrous tissue. An unanswered question is whether genetically programmed cardiomyocyte death in the form of apoptosis, which is observed in pressure overloaded hearts, contributes mechanistically to progressive remodeling and functional decompensation in cardiac hypertrophy. We previously observed stimulated expression of specific apoptosis genes in hearts undergoing physiological stress and recently found that ablation of the ischemia-regulated proapoptotic gene Bnip3 prevented postinfarction left ventricular remodeling in gene-targeted mice (*J Clin Invest*. 2007;117:2825–2833). Here, we show that a related proapoptotic factor, Nix, which is strikingly induced in pathological cardiac hypertrophy, contributes to adverse remodeling of pressure overload and genetic cardiac hypertrophies. Using mouse models in which the *Nix* gene was ablated in either the whole animal or specifically in cardiac myocytes, we show that absence of Nix in the heart prevents ≈50% of apoptotic cardiomyocyte cell death in response to pressure overload or genetically stimulated hypertrophy, restrains left ventricular dilation, and preserves contractile function, thereby preventing development of heart failure. These studies demonstrate feasibility for a general therapeutic strategy of "myocardial regeneration in reverse" by targeting specific stress-induced proapoptotic factors. In the case of pressure overload hypertrophy, functional decompensation can be prevented by minimizing apoptotic myocardial loss and the adverse remodeling that it causes by targeting hypertrophy-inducible Nix.

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