Cardiomyocyte NF-κB p65 promotes adverse remodelling, apoptosis, and endoplasmic reticulum stress in heart failure

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Aims
The role of nuclear factor (NF)-κB in heart failure (HF) is not well defined. We sought to determine whether myocyte-localized NF-κB p65 activation in HF exacerbatess post-infarction remodelling and promotes maladaptive endoplasmic reticulum (ER) stress.

Methods and results
Non-transgenic (NTg) and transgenic (Tg) mice with myocyte-restricted overexpression of a phosphorylation-resistant inhibitor of κB (IkBαS32A,S36A) underwent coronary ligation (to induce HF) or sham operation. Over 4 weeks, the remote myocardium of ligated hearts exhibited robust NF-κB activation that was almost exclusively p65 beyond 24 h. Compared with sham at 4 weeks, NTg HF hearts were dilated and dysfunctional, and exhibited hypertrophy, fibrosis, up-regulation of inflammatory cytokines, increased apoptosis, down-regulation of ER protein chaperones, and up-regulation of the ER stress-activated pro-apoptotic factor CHOP. Compared with NTg HF, Tg-IkBαS32A,S36A HF mice exhibited: (i) improved survival, chamber remodelling, systolic function, and pulmonary congestion, (ii) markedly diminished NF-κB p65 activation, cytokine expression, and fibrosis, and (iii) a three-fold reduction in apoptosis. Moreover, Tg-IkBαS32A,S36A HF hearts exhibited maintained expression of ER chaperones and CHOP when compared with sham. In cardiomyocytes, NF-κB activation was required for ER stress-mediated apoptosis, whereas abrogation of myocyte NF-κB shifted the ER stress response to one of adaptation and survival.

Conclusion
Persistent myocyte NF-κB p65 activation in HF exacerbatess cardiac remodelling by imparting pro-inflammatory, pro-fibrotic, and pro-apoptotic effects. p65 modulation of cell death in HF may occur in part from NF-κB-mediated transformation of the ER stress response from one of adaptation to one of apoptosis.

Keywords
NF-κB • Heart failure • Cardiac remodelling • Apoptosis • ER stress

1. Introduction
The transcription factor nuclear factor (NF)κB regulates genes that coordinate stress, growth, and inflammatory responses.¹,² NF-κB also influences cell survival and can induce either pro- or anti-apoptotic genes depending on the cell type and stimulus.³ In the endoplasmic reticulum (ER) stress response, for example, NF-κB comprises an important signal for an alarm phase that ultimately induces apoptosis in the face of prolonged ER stress,⁴ transforming an initially compensatory mechanism into a maladaptive one. The NF-κB family has five subunits—p65, RelB, c-Rel, p50, and p52—

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is in part regulated by the constituent homo- or heterodimers formed.

Chronic inflammation is a hallmark of heart failure (HF) and is a predictor of overall prognosis. Failing myocardium exhibits augmentation of both pro-inflammatory cytokine expression and NF-κB activation. Circulating monocytes in HF are also pathologically stimulated with augmented NF-κB activity. Despite the observation that both myocardium and inflammatory cells in HF demonstrate enhanced NF-κB, the pathophysiological role of NF-κB, whether it is protective or detrimental, and the importance of cell-type specificity are not well defined. Studies of post-infarction left ventricular (LV) remodelling in mice with targeted p50 deletion have yielded conflicting results. Two studies suggested that p50 exacerbates post-infarction remodelling and mortality, whereas another indicated the opposite—that p50 is cardioprotective and alleviates remodelling. Importantly, none of these studies examined the degree of p50 subunit activation in wild-type (WT) HF or the importance of myocyte (vs. non-myocyte) NF-κB. Accordingly, we tested the hypothesis that chronic NF-κB p65 activation in myocytes is pro-apoptotic and exacerbates post-infarction remodelling by using mice with myocyte-restricted transdominant expression of a mutant phosphorylation-resistant IkBα (IkBαS32A,S36A). IkBα is the primary regulator of the p65/p50 heterodimer and masks the nuclear localization sequence of p65; hence, IkBαS32A,S36A transgenic (Tg) mice are ideally suited for evaluating the effects of sustained myocyte p65 activation.

2. Methods

All studies were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication (NIH) 85-23, revised 1996). Local approval was given by the University of Louisville Institutional Animal Care and Use Committee (IACUC #08092). Additional methodological details are also provided in the Supplementary material online.

2.1 Mouse models

Male mice (10–22 weeks, 25–30 g) with myocyte-specific expression (α-MyHC promoter) of a transdominant mutant human IkBα with serine residues at positions 32 and 36 replaced by alanine (Tg-IkBαS32A,S36A) were used. These mice have been characterized previously and do not exhibit a baseline cardiac phenotype. The background strain is C57BL/6; non-transgenic (NTg) littermates were used as controls.

2.2 Coronary ligation

Left coronary artery ligation or sham operation was performed in mice as previously described and the mice were followed for 28 days after surgery. The total mice used were: NTg, n = 34; Tg-IkBαS32A,S36A, n = 33.

2.3 Echocardiography

Mouse echocardiography was performed using a Philips Sonos 5500 machine and 15 MHz linear array transducer as described previously.

2.4 Infarct size

Infarct size was measured 4 weeks after coronary ligation via morphological evaluation of freshly harvested LV tissue and image analysis. After tissue harvest and dissection, the LV was quickly sectioned into five transverse slices and photographed with a digital camera. The epicardial circular cross-section for the well-demarcated scar in each slice was determined post hoc by videoplanimetry (NIH Image) and summated for all slices, and then normalized to total LV circumference measured in all slices. Infarct (scar) size was expressed as a percentage of total LV.

2.5 Isolated cardiomyocyte studies and cell transfection

H9c2 cells (ATCC) in serum-free DMEM media were seeded in 100 mm tissue culture dishes and transfected for 24 h with the plasmid DNA (5 μg/dish) using Transfectin transfection reagent (BioRad) as described previously. In some protocols, cells were also treated with either recombinant mouse tumour necrosis factor (TNF) (20 ng/mL, BD Biosciences) or the ER stress inducer tunicamycin (TM, 10 μg/mL) for different time periods. Expression plasmids for NF-κB subunits p65 and p50 were purchased from Panomics. The expression plasmid for the dominant-negative IKKβ kinase mutant (KM) was a generous gift from Dr Hiroyasu Nakano (Juntendo University School of Medicine, Japan). Calcium-tolerant adult mouse cardiomyocytes were isolated using modified Langendorff perfusion and collagenase digestion, and seeded in supplemented DMEM media as described previously.

2.6 Western immunoblotting and electrophoretic mobility shift assay

Non-infarcted tissue was used for molecular analyses. Total protein extraction, SDS–PAGE western blotting, and immunodetection using electro-chemiluminescence were performed as previously described using commercially available antibodies. NF-κB subunits p65 and p50 were also measured in nuclear extracts using a commercially available ELISA kit (Panomics). NF-κB DNA-binding activity and subunit composition was quantified by electrophoretic mobility shift assay (EMSA) and gel super-shift as described previously.

2.7 Quantitative real-time PCR

Total RNA extraction from LV tissue, cDNA synthesis, and quantitative real-time PCR were performed as described previously. Relative levels of mRNA transcripts for atrial natriuretic factor (ANF), connective tissue growth factor (CTGF), TNF, interleukin (IL)-1β, and IL-6 were determined using primer pairs previously detailed and normalized to GAPDH expression using the ΔΔCt comparative method.

2.8 Histological analysis

H&E and Masson’s trichrome stains were used to determine cardiomyocyte cross-sectional area and myocardial fibrosis. Apoptosis was assessed by terminal deoxytransferase-mediated dUTP nick-end labelling (TUNEL) using the ApopTag kit in Situ Detection Kit (Oncor) as described previously. Immunoreactivity or TUNEL positivity was quantified from at least 20 random fields by light microscopy.

2.9 Statistical analysis

For two-group comparisons, we used the unpaired two-sample t-test. For comparisons of more than two groups, we used one-way ANOVA if there was one independent variable and two-way ANOVA if there were two independent variables (e.g. genotype and ligation status). To adjust for multiple comparisons, we performed a Bonferroni post-test. Pair-wise comparisons were made between sham groups across genotypes, sham vs. HF within each genotype, and HF groups across genotypes. A P-value of <0.05 was considered significant. Animal survival was evaluated by the Kaplan–Meier method, and the log-rank test was used to compare survival curves between NTg sham and HF, Tg sham and HF, and between NTg HF and Tg HF. Continuous data are summarized as mean ± SD.
3. Results

3.1 NF-κB p65 is persistently activated in the remodelling heart

WT C57BL/6 mice underwent coronary ligation or sham operation and NF-κB activation was evaluated in remote myocardium 4 weeks after surgery (Figure 1A–C). EMSA revealed robust NF-κB activation in failing hearts compared with sham. Gel supershift revealed that p65 was the major subunit activated along with a minor amount of RelB, but negligible p50, despite probing with two different p50 subunit antibodies (Figure 1A). The specificity of NF-κB DNA binding was confirmed using cold competition with a 100-fold unlabelled consensus sequence (see Supplementary material online, Figure S1A). An alternative measurement for nuclear p65 and p50 using ELISA confirmed significant translocation of p65, but not p50, in failing hearts over sham (Figure 1B). Moreover, immunoblotting for NF-κB subunits in cytosolic and nuclear extracts revealed augmented p65 (and RelB) nuclear translocation in failing hearts but with diminished p50 translocation and no change in c-Rel (Figure 1C).

To exclude the possibility that the lack of p50 detection was related to suboptimal antibody fidelity, H9c2 cells, an embryonic cardiomyoblast line, were transiently transfected with the expression plasmid encoding human p50 in the presence or absence of TNF-α (20 ng/mL) for 30 min (representative results from four independent experiments). (D) NF-κB DNA-binding activity and subunit composition by EMSA, gel supershifts, and immunoblotting on NEs isolated from H9c2 cells transiently transfected with human NF-κB p50 in the presence or absence of TNF-α (20 ng/mL) for 30 min (representative results from four independent experiments). (E) NF-κB p65 and p50 subunit-specific ELISA in NEs isolated from non-infarcted myocardium of WT animals at indicated times after coronary ligation. *P < 0.05 vs. sham; #P < 0.05 vs. naïve; n = 6–8/group unless otherwise indicated. FP, free probe.
translocation increased. However, at later time points, nuclear p65 levels increased further and remained persistently elevated, whereas p50 levels returned to baseline.

### 3.2 Myocyte NF-κB abrogation improves post-infarction survival and alleviates LV remodelling

Echocardiography revealed no baseline differences in LV structure or systolic function between NTg and Tg-IκBαS32A,S36A mice (data not shown). The Kaplan–Meier survival curves (Figure 2A) revealed significantly increased mortality for NTg HF mice over sham at 28-day post-infarction but only a non-significant trend (P = 0.088) towards increased mortality in Tg-IκBαS32A,S36A HF mice compared with sham. Tg-IκBαS32A,S36A HF mice exhibited improved survival in comparison to NTg HF. At baseline, there was a four-fold increase in IκBα protein expression in Tg-IκBαS32A,S36A hearts when compared with NTg hearts (see Supplementary material online, Figure S2A). In NTg HF hearts, IκBα levels were markedly diminished compared with NTg sham, consistent with augmented NF-κB activation (see Supplementary material online, Figure S2B). IκBα protein levels, while still relatively high, also decreased in Tg-IκBαS32A,S36A failing hearts when compared with Tg-IκBαS32A,S36A sham. EMSA of nuclear extracts from failing hearts revealed robust activation of NF-κB in NTg HF vs. sham (Figure 2B). In Tg-IκBαS32A,S36A mice, although NF-κB was also activated in HF, such activation was markedly reduced when compared with NTg. Immunoblotting of cytosolic and nuclear extracts revealed that as in WT HF, p65 was the primary subunit activated in Tg-IκBαS32A,S36A HF hearts with negligible p50 (Figure 2C).

Figure 2D depicts representative short-axis LV sections and M-mode echocardiograms and Table 1 presents the group echocardiographic and gravimetric data. There was LV dilatation (increased LV end-diastolic volume and LV end-systolic volume) and systolic dysfunction (reduced LV ejection fraction and Vcf) in both NTg and Tg-IκBαS32A,S36A HF mice. However, compared with NTg HF, LV dilatation and dysfunction were attenuated in Tg-IκBαS32A,S36A HF. LV, right ventricle (RV), and lung weight normalized to tibia length (TL).
Table 1: Echocardiographic and gravimetric data from NTg and Tg-IkBα<sup>S32A,S36A</sup> mice

<table>
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<tr>
<th></th>
<th>NTg</th>
<th>Tg-IkBα&lt;sup&gt;S32A,S36A&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Sham</td>
<td>HF</td>
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<td></td>
<td>Sham</td>
<td>HF</td>
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<tr>
<td>HR (b.p.m.)</td>
<td>522 ± 29</td>
<td>521 ± 50</td>
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<tr>
<td>LVEDD (mm)</td>
<td>4.0 ± 0.4</td>
<td>5.1 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.0 ± 0.2</td>
<td>4.2 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>FS (%)</td>
<td>49 ± 3</td>
<td>18 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LVEDV (µL)</td>
<td>46 ± 21</td>
<td>82 ± 24&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LVESV (µL)</td>
<td>16 ± 9</td>
<td>56 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LVEF (%)</td>
<td>66 ± 7</td>
<td>37 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>AWT (mm)</td>
<td>0.66 ± 0.09</td>
<td>0.88 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>V&lt;sub&gt;c&lt;/sub&gt; (circ/s)</td>
<td>10.4 ± 1.7</td>
<td>3.9 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LV weight/TL (mg/mm)</td>
<td>3.7 ± 0.2</td>
<td>4.8 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>RV weight/TL (mg/mm)</td>
<td>1.0 ± 0.1</td>
<td>1.6 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lung weight/TL (mg/mm)</td>
<td>5.9 ± 0.57</td>
<td>9.3 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
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HF, heart failure; HR, heart rate; LV, left ventricular; EDD, end-diastolic diameter; EDV, end-diastolic volume; ESD, end-systolic diameter; ESV, end-systolic volume; FS, fractional shortening; EF, ejection fraction; AWT, anterior wall thickness at end-diastole; V<sub>c</sub>, velocity of circumferential fibre shortening; TL, tibia length; RV, right ventricle. n = 7–12/group.

<sup>a</sup>P < 0.05 vs. respective sham.

<sup>b</sup>P < 0.05 vs. NTg HF.

all significantly increased in NTg HF hearts over sham, indicating biventricular hypertrophy and pulmonary congestion (secondary to elevated LV filling pressure) (Table 1). LV/TL and RV/TL were similar between Tg-IkBα<sup>S32A,S36A</sup> HF and NTg HF mice. However, lung/TL was significantly decreased in Tg-IkBα<sup>S32A,S36A</sup> HF mice, indicating less pulmonary congestion and lower filling pressures. Notably, infract size at 4 weeks was equivalent in NTg and Tg-IkBα<sup>S32A,S36A</sup> HF mice (Figure 2E), suggesting that these changes did not result from different degrees of injury but rather to subsequent differences in remodelling.

3.3 Myocyte NF-κB abrogation prevents pro-inflammatory cytokine expression and fibrosis without impacting hypertrophy in HF

Consistent with the gravimetric data, both NTg and Tg-IkBα<sup>S32A,S36A</sup> HF hearts exhibited increased ANF gene expression and myocyte diameter when compared with sham, consistent with cardiac hypertrophy (Figure 3A). However, there were no differences in these parameters between NTg and Tg HF. Collagen deposition in both remote and border zone myocardium was significantly increased in the failing heart (Figure 3B). The degree of fibrosis was markedly attenuated, however, in Tg-IkBα<sup>S32A,S36A</sup> HF when compared with NTg HF, and not different from Tg-IkBα<sup>S32A,S36A</sup> sham. Moreover, cardiac gene expression of CTGF, a pro-fibrotic matrix-associated protein, was increased five-fold in NTg HF over sham, but such up-regulation was completely prevented in Tg-IkBα<sup>S32A,S36A</sup> HF. As NF-κB is a potent inducer of inflammation, we next examined gene expression of the pro-inflammatory cytokines TNF, IL-1β, and IL-6. Each of these cytokines was markedly up-regulated in NTg HF hearts over sham (Figure 4). In contrast, Tg-IkBα<sup>S32A,S36A</sup> HF hearts exhibited either diminished (TNF) or unchanged (IL-1β and IL-6) cytokine expression compared with sham.

3.4 Myocyte NF-κB abrogation decreases apoptosis and promotes adaptive ER stress responses in HF

We have previously demonstrated that sustained p65 overexpression is pro-apoptotic in H9c2 cardiomyocytes, and that, along with NF-κB, other mediators of the alarm phase of ER stress—p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK)—are activated in failing hearts. Hence, we evaluated the effects of myocyte NF-κB abrogation on apoptosis and ER stress. As shown in Figure 5A, TUNEL-positive nuclei were markedly increased in NTg HF hearts over NTg sham but significantly attenuated in Tg-IkBα<sup>S32A,S36A</sup> HF, which in turn was not significantly augmented over Tg-IkBα<sup>S32A,S36A</sup> sham.

The adaptive phase of ER stress is characterized by increased expression of the ER chaperone proteins Grp78, Grp94, and calreticulin that help stabilize protein folding intermediates. In the face of excessive ER stress, however, alarm phase mediators (e.g., NF-κB, p38 MAPK, and JNK) are activated that ultimately up-regulate the pro-apoptotic transcription factor CHOP (GADD153) and proteolytically process caspase-12 to induce cell death. Figure 5B shows protein expression of Grp78, Grp94, and CHOP in NTg and Tg-IkBα<sup>S32A,S36A</sup> sham and HF hearts. When compared with sham, NTg HF hearts exhibited significantly reduced expression of Grp78 and Grp94 and robust up-regulation of CHOP demonstrating that in established HF, there is a preponderance of alarm phase ER stress responses associated with the augmented apoptosis. In contrast, Tg-IkBα<sup>S32A,S36A</sup> HF hearts maintained the expression of Grp78 and Grp94 and exhibited no significant change in CHOP when compared with sham, indicating preservation of adaptive ER stress responses.

3.5 NF-κB activation is required for ER stress-mediated apoptosis in cardiomyocytes

As shown in Figure 6A, prolonged (≥8–24 h) stimulation of H9c2 cardiomyocytes with the classical pharmacological ER stress inducer TMN increased phosphorylation and degradation of IkBα and NF-κB nuclear translocation. This was associated with phosphorylation of PERK and JNK1/2, up-regulation of Grp78, Grp94, and CHOP, and increased cleavage of PARP and caspase-3, all consistent with activation of ER stress and ER stress-mediated apoptosis (Figure 6B). To determine the role of NF-κB in ER stress-mediated cell death, the experiments were repeated after transfection and overexpression of a dominant-negative (DN) IKKβ mutant. In separate validation studies, DN IKKβ was highly effective in blocking NF-κB activation in response to TNF stimulation (see Supplementary material online, Figure S3). As seen in Figure 6B, DN IKKβ overexpression prevented IkBα degradation but had no effect on expression of Grp78, Grp94, and CHOP or the phosphorylation of PERK. However, there was suppression of JNK1/2 phosphorylation and ER stress-mediated apoptosis with considerably less cleavage of PARP and caspase-3. The significance of JNK1/2 phosphorylation is shown in Figure 6C—pharmacological JNK inhibition with SP600125 during ER stress diminished.
IKB degradation and CHOP up-regulation and similarly attenuated PARP and caspase-3 cleavage.

We next assessed ER stress-mediated cell death in adult NTg and Tg-IKBS32A,S36A cardiomyocytes. As seen in the phase contrast images and quantitation in Figure 6D, Tg-IKBS32A,S36A myocytes exhibited improved survival in response to 24 h of TM exposure when compared with NTg myocytes. As seen in Figure 6E, prolonged TM stimulation of NTg myocytes induced IKB degradation (indicative of NF-κB activation), up-regulation of the ER chaperones Grp78, Grp94, and calreticulin, and induction of CHOP and apoptosis suggested by a decrease in procaspase-12 (indicating increased proteolytic processing) and increased cleavage of PARP. In contrast, akin to the HF studies, Tg-IKBS32A,S36A myocytes exhibited augmented ER chaperones but with marked suppression of CHOP, procaspase-12 processing, and PARP cleavage indicating abrogation of ER stress-dependent apoptosis.

4. Discussion

We have demonstrated that chronic NF-κB activity in the murine failing heart is primarily related to p65 and not p50, and that sustained myocyte-localized p65 activation reduces survival and promotes post-infarction LV remodelling, imparting pro-fibrotic, pro-inflammatory, and pro-apoptotic effects. We have also shown that NF-κB is required for ER stress-mediated apoptosis in cardiomyocytes and that abrogation of myocyte NF-κB p65 in isolated cardiomyocytes...
or in the failing heart in vivo shifts the ER stress response to one of adaptation rather than apoptosis. The results establish multifaceted roles for myocyte NF-κB in general, and the p65 subunit in particular, related to inflammation, ER stress, and apoptosis that ultimately exacerbate cardiac remodelling and dysfunction in HF.

Failing hearts exhibit chronic activation of NF-κB and up-regulation of NF-κB-responsive genes.9–11 Although classically considered a pro-survival transcription factor, studies suggest that NF-κB serves as a control point that can induce either survival or death depending on the cell type and nature of the stimulus.3 Indeed, NF-κB can up-regulate both anti-apoptotic genes (TRAF1/2, cIAP-1/2, A1/Bfl-1, Bcl-XL, and cFLIP) and pro-apoptotic genes (Fas, FasL, DR4, DR5, TRAIL, and p53).3,24–26 Whether NF-κB confers primarily detrimental or beneficial effects in chronic HF is controversial.26 Using p50 null mice, two groups reported that p50 deficiency improved survival and ameliorated post-infarction remodelling, fibrosis, hypertrophy, and dysfunction,13,14 but did not attenuate inflammatory cytokine expression.14 In contrast, a third study using magnetic resonance imaging reported the opposite—that p50 deletion aggravated LV remodelling, dysfunction, hypertrophy, and fibrosis and increased inflammation.15 Although the underlying reasons for this disparity are unclear, none of these studies determined the status of NF-κB subunit activation in chronically remodelled myocardium (and hence the biological importance of p50 activity) in WT HF. Also, unclear is the relative importance of myocyte vs. non-myocyte (e.g. inflammatory cell) responses as somatic p50 null mice were used. Cell type-specific effects may be of importance in HF, given that adoptive transfer models with selective p50 deficiency in the bone marrow (and not in cardiomyocytes) exhibit cardioprotection during acute ischaemia/reperfusion.27

In contrast to our results in post-infarction remodelling, two recent studies suggest that cardiomyocyte-localized NF-κB plays a protective role in pressure-overload hypertrophy in that cardiomyocyte-specific deletion of either IKKβ or the NF-κB essential modulator (NEMO and IKKγ) exacerbates apoptosis, hypertrophy, fibrosis, and the development of HF after transverse aortic constriction.28,29 These results may relate to differences in experimental model and serve to underscore the stimulus- and disease-specific effects of NF-κB. However, upstream IKKs also activate NF-κB-independent signalling pathways,30 many of which (e.g. p53 and FOXO3A) can also potentially influence remodelling. Moreover, in these studies of IKK ablation, the relative makeup of the NF-κB subunits was not quantified, and consequently, the role of subunit-specific responses in these effects remains unclear.

Our results demonstrate that after the initial 24 h during which both p65 and p50 translocate to the nucleus, NF-κB activity in non-infarcted murine myocardium is almost entirely p65. Given the primacy of nuclear p65 in chronically failing myocardium, and as there is known subunit specificity in the ensuing transcriptional

![Figure 5](https://academic.oup.com/cardiovascres/article-abstract/89/1/129/326710/135)

Myocyte-specific NF-κB abrogation attenuates apoptosis and exacerbates maladaptive ER stress in the failing heart. (A) Representative TUNEL stain histomicrograph depicting an apoptotic nucleus (arrow) from a non-transgenic (NTg) HF heart and the corresponding quantitation from NTg and Tg-κBαS32A,S36A sham and HF hearts. Scale bar = 15 μm. (B) Immunoblot analysis of ER stress proteins in NTg and Tg sham and HF hearts and corresponding densitometry. *P < 0.05 vs. respective sham; #P < 0.01 vs. NTg HF; n = 4–6/group.
responses.\textsuperscript{25} p65-specific gene targets are likely to have more important roles in cardiac remodelling. Indeed, whereas p65 generally induces gene transactivation, the p50 subunit is considered to inhibit rather than stimulate transcriptional activity in part because of an absence of TADs.\textsuperscript{2,5 – 7} Moreover, LPS stimulation of p50 mouse embryonic fibroblasts markedly increases nuclear p65 expression.\textsuperscript{15} Hence, the divergent experimental results with p50 ablation discussed above may be related to unrecognized differences in the degree of compensatory p65 activation and/or up-regulation in mice with p50 deletion. In our study, we specifically targeted myocyte-localized p65 via transdominant expression of phosphorylation-resistant IκBα, which masks the nuclear localization sequence of p65.\textsuperscript{17}

In our model, chronic NF-κB p65 activation in myocytes aggravated LV remodelling, dilatation, and dysfunction following myocardial infarction. Consistent with its central role in triggering inflammation, myocyte p65 activation was an absolute requirement for the up-regulation of the inflammatory cytokines TNF, IL-1β, and IL-6 in the failing heart. Indeed, Tg-IκBα\textsuperscript{S32A,S36A} HF mice actually exhibited reduced cytokine expression in comparison to sham; the myocyte localization of these effects is consistent with failing myocytes serving as robust sources of inflammatory cytokines. Moreover, suppression of myocardial cytokine expression was associated with amelioration of interstitial fibrosis, consistent with the known pro-fibrotic effects of pro-inflammatory mediators.\textsuperscript{8}

Although LV chamber size was significantly smaller in Tg-IκBα\textsuperscript{S32A,S36A} HF mice when compared with NTg HF mice, the abrogation of myocyte NF-κB in these mice did not impact the degree of cardiac hypertrophy, as assessed by complementary approaches of gravimetry, ANF expression, and myocyte size. This observation is somewhat surprising given that in neonatal cardiomyocytes NF-κB activation is required for G-protein-coupled receptor

**Figure 6** NF-κB activation mediates ER stress-induced apoptosis in cardiomyocytes. (A) H9c2 cardiomyocytes were treated with vehicle (V) or TM (10 μg/mL) for the indicated times and NF-κB activation was evaluated by immunoblotting for total and phosphorylated IκBα and DNA-binding by EMSA. (B) H9c2 cells were transiently transfected either with 5 μg of empty plasmid as a control or IKKβ KM expression vector for 24 h before treating with TM (10 μg/mL) for the indicated times and cell lysates were prepared. Total lysates (25–30 μg) were then immunoblotted for ER stress markers and mediators of apoptosis. (C) H9c2 cardiomyocytes were treated as in (A) in the presence and absence of the JNK inhibitor SP600125 (20 μM) and total cell lysates (25–30 μg) were immunoblotted for ER stress and apoptotic markers. (D) Equal numbers of adult mouse cardiomyocytes isolated from naive NTg and Tg-IκBα\textsuperscript{S32A,S36A} mice were treated either with vehicle (DMSO, V) or TM (10 μg/mL) for 24 h. Shown are the representative bright field images before and after treatment and quantitation of remaining rod-shaped myocytes at 24 h. (E) Adult mouse cardiomyocytes were isolated from naive NTg and Tg hearts and plated on collagen coated dishes for 6 h before treating with TM (10 μg/mL) for the indicated times. Total lysates were then immunoblotted for ER stress markers and mediators of apoptosis. Results in each panel are representative of three to five independent experiments.
agonist-induced hypertrophy, but serves to highlight important differences between responses in isolated cells vs. in vivo pathology. Given the absence of changes in myocyte hypertrophy, an alternate mechanism for the marked differences in LV remodelling between Tg- IkBa32A,536A HF and NTg HF may be attributed to differences in the rates of apoptosis and cardiac cell loss, together with attendant changes in replacement fibrosis. Indeed, Tg- IkBa32A,536A HF hearts exhibited a three-fold reduction in the apoptotic rate, suggesting that p65 activation has a substantial impact on apoptosis. These results are consistent with our prior work, demonstrating that NF-kB activation in H9c2 cardiomyocytes is pro-apoptotic in a TNF receptor (R)1-dependent manner. In that study, we also showed that TNFR1 signalling aggravated, whereas TNFR2 signalling ameliorated, post-infarction remodelling, apoptosis, inflammation, and NF-kB activation in vivo. Taken together with our current results, differential modulation of myocyte NF-kB activity likely contributes to the divergent effects of the two TNF receptors in HF.

One mechanism by which NF-kB can influence cell death is through the ER stress response, which is known to be activated in HF. Cellular stress (e.g. altered redox state) results in the accumulation of unfolded proteins in the ER and the release of ER chaperones Grp78, Grp94, and calreticulin. The release of ER chaperones initially triggers adaptive responses to restore homeostasis and reduce ER protein load including activation of the ER transmembrane proteins PERK, Ire1, and ATF6, suppression of global mRNA translation, and selective up-regulation of ER chaperones and proteins involved in the retrograde transport of misfolded proteins from the ER to the cytosol. However, in the face of excessive ER stress, an alarm phase is initiated by the activation of several kinases including p38MAPK, JNK, and IKK, which subsequently results in NF-kB nuclear translocation. The end-outcome of alarm gene activation is cell death secondary to the induction of pro-apoptotic transcription factors such as CHOP, and, in rodents, the proteolytic cleavage of caspase-12.

Our results confirm that in H9c2 cardiomyocytes, a cell type that does not express p50 (Figure 1), TM-mediated ER stress induces PERK phosphorylation and the up-regulation of ER chaperones, but that prolonged exposure phosphorylates JNK1/2, up-regulates CHOP, and causes apoptosis.

In the remote myocardium of NTg failing hearts, 4 weeks after infarction, there was down-regulation of the ER chaperones Grp78 and Grp94, but very obvious and robust expression of CHOP accompanying the increased apoptosis, indicating preponderance of the alarm phase of ER stress. However, attenuation of NF-kB p65 activation in Tg- IkBa32A,536A failing hearts was accompanied by persistent expression of Grp78 and Grp94 (suggesting greater ER protein folding capacity) and no change in CHOP, suggesting that NF-kB serves as a switch to convert the ER stress response from one of adaptation to alarm in chronic HF. Indeed, in TM-treated H9c2 cells, NF-kB inhibition with IKKβ KM overexpression prevented both JNK1/2 phosphorylation and apoptosis. Moreover, Tg- IkBa32A,536A adult cardiomyocytes were resistant to TM-induced ER stress-related apoptosis, exhibiting maintenance of ER chaperones, suppression of CHOP, and attenuation of procaspase-12 processing, consistent with the in vivo results in Tg- IkBa32A,536A HF hearts. These results support a model in which p65 activation is the primary driver of detrimental ER stress responses and ER stress-mediated apoptosis in HF. Notably, these results are consistent with a recent study in rat insulinoma cells, demonstrating that NF-kB inhibition prevents thapsigargin-induced ER stress-mediated cell death but not nitric oxide-mediated apoptosis. Taken together, our results establish that a complex interplay between cytokine-mediated inflammatory responses, ER stress, and apoptosis underlies the detrimental effects of myocyte NF-kB activation in the failing heart.

In summary, NF-kB activation in the murine failing heart is primarily the p65 subunit, with negligible p50. Chronic augmentation of myocyte-localized p65 activity is detrimental in post-infarction HF, increases mortality, and aggravates pathological remodelling and dysfunction with pro-inflammatory, pro-fibrotic, and pro-apoptotic effects. The modulation of cell death in the failing heart by NF-kB p65 occurs at least in part by its regulation of the alarm phase of the ER stress response, as it is required for ER stress-mediated cardiomyocyte apoptosis. Indeed, in failing myocytes, NF-kB p65 activity may serve as a nodal point in the transformation of the ER stress response from one of adaptation to one of cell death. Hence, targeted blockade of p65 in the heart may be a useful therapeutic strategy to maintain homeostatic responses to ER stress and ameliorate myocyte loss in the remodelling heart.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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