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NF κ B promotes oxidative stress-induced necrosis and ischemia/reperfusion injury by inhibiting Nrf2-ARE pathway

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Abstract

In this study, we identified an unexpected pro-cell death role for NF κ B in mediating oxidative stress-induced necrosis, and provide new mechanistic evidence that NF κ B, in cooperation with HDAC3, negatively regulates Nrf2-ARE anti-oxidative signaling through transcriptional silencing. We showed that genetic inactivation of NF κ B-p65 inhibited, whereas activation of NF κ B promoted, oxidative stress-induced cell death and HMGB1 release, a biomarker of necrosis. Moreover, NF κ B-luciferase activity was elevated in cardiomyocytes after simulated ischemia/reperfusion (sI/R) or doxorubicin (DOX) treatment, and inhibition of NF κ B with Ad-p65-shRNA or Ad-I κ B α M diminished sI/R- and DOX-induced cell death and HMGB1 release. Importantly, NF κ B negatively regulated Nrf2-ARE activity and the expression of antioxidant proteins. Mechanistically, co-immunoprecipitation revealed that p65 was required for Nrf2-HDAC3 interaction and transcriptional silencing of Nrf2-ARE activity. Further, the ability of HDAC3 to repress Nrf2-ARE activity was lost in p65 deficient cells. Pharmacologic inhibition of HDACs or NF κ B with trichostatin A (TSA) or BMS-345541, respectively, increased Nrf2-ARE activity and promoted cell survival after sI/R. *In vivo*, NF κ B transcriptional activity in the mouse heart was significantly elevated after ischemia/reperfusion (I/R) injury, which was abolished by cardiomyocyte-specific deletion of p65 using *p65^{fl/fl}*Nkx2.5-Cre mice. Moreover, genetic ablation of p65 in the mouse heart attenuated myocardial infarct size after acute I/R injury and improved cardiac remodeling and functional recovery after chronic myocardial infarction. Thus, our results identified NF κ B as a key regulator of oxidative stress-induced necrosis by suppressing the Nrf2-ARE antioxidant pathway through an HDAC3-dependent mechanism. This study also revealed a new pathogenic role of NF κ B in cardiac ischemic injury and pathological remodeling.

Graphical Abstract

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Declarations of interest
None.

Keywords

Oxidative stress; necrosis; NFκB; Nrf2; HDAC3; ischemia/reperfusion injury

Introduction

The NFκB/Rel family of transcription factors regulate a wide range of cellular processes such as proliferation, differentiation, immune responses, inflammation, and cell survival/death^{1,2}. NFκB is composed of homo- and heterodimeric complexes of members of the Rel family of proteins, including p65 (RelA), p105/p50, p100/p52, RelB, and c-Rel. The best-studied and most abundant component of these complexes is the p65/p50 heterodimer. In unstimulated cells, NFκB binds to cytosolic inhibitory proteins, IκBs, mediating cytoplasmic retention. In the canonical activation pathway, stimulation with inflammatory cytokines (*e.g.*, tumor necrosis factor-α) or bacterial products (*e.g.*, lipopolysaccharide) leads to phosphorylation of IκBα on serines 32 and 36 by IKK, the IκB kinase³. This results in the ubiquitination and degradation of IκBα and subsequent release of NFκB for nuclear translocation where NFκB acts as a sequence-specific, DNA binding transcription factor^{4,5}. In addition to “typical” activators such as TNFα and LPS, NFκB can also be activated by “atypical” activators, such as ultraviolet light, ionizing radiation, chemotherapeutic compounds, and oxidative stress^{3,6}. This atypical mechanism shows slower kinetics of NFκB nuclear translocation and may involve a pathway distinct from that utilized by inflammatory cytokines. For example, it has been shown that UV and doxorubicin activate NFκB in an IKK-independent manner^{7,8}. In addition, oxidative stress has been shown to activate NFκB through induction of tyrosine phosphorylation of IκBα⁹.

Most of the biological actions of NFκB have been ascribed to its well-known role as a transcriptional activator. For example, the anti-cell death function of NFκB has been associated with its role in promoting transcriptional expression of pro-survival factors^{10,11}. However, NFκB may also act as a transcriptional repressor that negatively regulates gene

expression by recruiting corepressors such as histone deacetylases (HDACs)^{12,13}. It is well-established that HDACs inhibit gene transcription by deacetylating histone core proteins, which compacts the nucleosome sterically impairing access of transcription factors to DNA^{14,15}. Moreover, HDACs can also regulate gene transcription by modifying nonhistone DNA binding factors. Indeed, it has been reported that HDACs inhibit gene transcription by interacting with several transcription factors^{12,16–18}.

The anti-cell death role of NFκB has been extensively investigated. The murine *Rela* (*p65*) gene knockout leads to embryonic lethality with massive apoptosis in the embryonic liver¹⁹. Further studies have demonstrated that NFκB has an anti-apoptotic effect in response to diverse stimuli, including inflammatory cytokines, hypoxia, ionizing radiation, and chemotherapeutic drugs^{19,20–22}. Intriguingly, emerging evidence suggests that NFκB may also have a pro-cell death function under some circumstances^{23–25}. These effects are less well characterized, but NFκB has been implicated as a repressor of pro-survival genes and/or inducer of pro-death genes. Indeed, *Rela* (*p65*) deficient MEFs displayed increased resistance to UV induced cell death, along with increased expression of antiapoptotic genes such as Bcl-xL and X-IAP²⁵. Moreover, inactivation of NFκB reduced cell death induced by chronic H₂O₂ exposure and inhibited expression of pro-death genes such as TNFα and FasL²⁶. However, the molecular mechanism by which NFκB mediates the pro-cell death function remains elusive.

NFκB is activated after cardiac ischemia-reperfusion injury in animal models as well as in patients with heart failure^{27–29}. Clinical studies showed that reduced NFκB activity is correlated with improved myocardial remodeling and functional recovery^{30,31}. Cardiac-specific overexpression of a dominant-negative IκBα³² or genetic ablation of NFκB p65³³ reduced infarct size and preserved heart function in response to I/R, indicating that the activation of NFκB within the cardiomyocyte is critical to the pathogenesis of cardiac I/R injury. Pharmacological inhibitors of NFκB also provided cardio-protection against cardiac I/R injury by reducing infarct size and improving functional recovery^{29,34,35}. However, the mechanisms underlying the beneficial effects of NFκB inhibition in the setting of cardiac I/R injury remain incompletely understood.

In this study, we sought to determine the role of NFκB in oxidative stress-induced cell death *in vitro* and the functional implications in cardiac I/R injury *in vivo*. We provide new mechanistic evidence that NFκB p65 promotes oxidative stress-induced necrosis by suppressing the NRF2-ARE antioxidant pathway through HDAC3-dependent transcriptional silencing. Moreover, using a cardiomyocyte-specific p65 knockout mouse model, our results demonstrate a new pathogenic role of NFκB p65 in mediating cardiac I/R injury, myocardial cell death, and pathological remodeling.

Materials and Methods

Reagents

Mouse TNFα was from R&D Systems. tert-butylhydroperoxide, hydrogen peroxide, cycloheximide, BMS-345541, and TSA were from Sigma. Doxorubicin hydrochloride was from Cayman Chemical. Propidium iodide and Hoechst 33342 were from Invitrogen. The

following antibodies were used: anti- α -tubulin (3873), anti-HMGB1 (3935), anti-PARP (9532), anti-HO-1 (82206), anti-IKK β (2678), and anti-HDAC3 (3949) were from Cell Signaling Biotechnology (Beverly, MA); Anti-p65 (sc-372), Anti-Nrf2 (sc-722), anti-NQO1 (sc-393736), anti-I κ B α (sc-847), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

Primary neonatal rat cardiomyocytes were prepared from hearts of 1- to 2-day-old Sprague-Dawley rat pups as previously described³⁶. After separation from fibroblasts, enriched cardiomyocytes were plated on 1% gelatin-coated 12-well plates for luciferase assays or on 6-cm-diameter dishes for all other experiments. Cells were grown in M199 medium containing 100 U of penicillin-streptomycin/ml and 2 mM L-glutamine without serum for 24 h before infection. Mouse embryonic fibroblasts derived from p65^{+/+} and p65^{-/-} mice were kindly provided by David Baltimore at California Institute of Technology. To induce oxidative stress, cells were treated with 50–100 μ M tert-butylhydroperoxide, hydrogen peroxide, or 10 μ M Doxorubicin for up to 12 h. For simulated ischemia/reperfusion (sI/R), ischemia was imposed by a buffer exchange to ischemic solution (in mM: 20 deoxyglucose, 125 NaCl, 8 KCl, 1.2 KH₂PO₄, 1.25 MgSO₄, 1.2 CaCl₂, 6.25 NaHCO₃, 5 sodium lactate, 20 HEPES, pH 6.6) and placing the plates within a humidified chamber equilibrated with 95% N₂ and 5% CO₂. After 6 h of simulated ischemia, reperfusion was initiated by buffer exchange to normoxic culture medium in 95% room air and 5% CO₂ for 12 h. Controls incubated in normoxic culture medium in 95% room air and 5% CO₂ were prepared in parallel for each condition.

Cell death assays

Cell death was measured using a Cell Meter Apoptotic and Necrotic Detection kit (ATT Bioquest, Sunnyvale, CA, USA) as previously described³⁷. Briefly, cells were incubated at 37 °C for 30 min with Apopxin Green for detection of phosphatidylserine on cell surface, PI or 7-ADD for labeling the nucleus of cells with membrane rupture, and CytoCalcein for labeling live cell cytoplasm. Cell death was then analyzed with an EVOS FL digital fluorescence microscope (AMG) or a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cells with chromatin condensation were visualized by Hoechst 33342 (Invitrogen, Waltham, MA, USA) staining. Cell viability was also assessed using the Muse Count & Viability assay kit (Millipore, Billerica, MA, USA). In brief, cells were trypsinized, washed, and incubated with the Muse Count & Viability reagent, and cell viability was quantified on a Muse cell analyzer (Millipore).

Adenoviral vectors

Adenoviral vectors expressing p65 shRNA or a scrambled shRNA were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's protocol. Ad- β gal, Ad-IKK β , Ad-p65, and Ad-I κ B α M have been described previously³⁸. Ad-NF κ B luciferase reporter was obtained from Vector Biolabs (Philadelphia, PA, USA). Ad-HDAC3 was from SignaGen Laboratories. Ad-Nrf2 and Ad-shNrf2 were provided by Taixing Cui at University of South Carolina. Adenoviral infections were performed as described previously at a multiplicity of infection of 10 to 50 plaque forming

units per ml. Cells were harvested 24 h after infection followed by western blot analysis, luciferase assay, or cell death assays.

Luciferase activity assays

Nrf2-ARE transcriptional activity was measured using a Nrf2-ARE reporter kit (BPS Bioscience) according to the manufacturer's instructions. Briefly, cells are transfected with an ARE luciferase reporter vector, which contains a firefly luciferase gene under the control of multimerized ARE responsive elements located upstream of a minimal promoter. A renilla luciferase vector was included in all samples to control for transfection efficiency. Reporter assays were performed using a dual-luciferase (firefly-renilla) reporter gene assay system. NF κ B luciferase reporter assay was performed as we previously described³⁷. Cells transduced with the NF κ B luciferase reporter were washed in PBS and then resuspended in lysis buffer (100 mM KH₂PO₄, pH 7.8, 0.5% Nonidet P-40, and 1 mM DTT). Cellular lysates were centrifuged at 3 000 g for 10 min at 4 °C and supernatants were assayed in the luciferase assay buffer (100 mM Tris-HCl, pH 7.8, 10 mM Magnesium acetate, 1 mM EDTA, 1 mM DTT, 2 mM ATP, and 1 mM luciferin). Luminescence was determined with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Immunoprecipitation

Immunoprecipitation was performed as described previously^{39,40}. Cells were lysed at 4 °C in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 0.5% NP-40) containing protease and phosphatase inhibitor cocktail (Roche, Indianapolis, IN, USA). Whole cell lysates were cleared by centrifugation at 18 000 g for 10 min and then incubated with 2 μ g antibodies as indicated and protein A/G-PLUS agarose beads (Santa Cruz Biotechnologies) overnight at 4 °C. The beads were washed extensively with wash buffer (0.3% NP-40 in PBS), and the proteins were resolved on an 8–12% SDS-PAGE for subsequent western blotting.

Western blotting

Cells were lysed using RIPA buffer (20 mM Tris pH 7.5, 200 mM NaCl, 1% Triton-X 100, 2 mM DTT, 5 mM p- nitrophenyl phosphate, 2 mM sodium orthovanadate, 1X protease inhibitor cocktail [Roche]). Western blotting followed by enhanced chemiluminescence detection was performed as previously described⁴⁰.

Mouse models and procedures

Rela (*p65*) *loxP*-targeted (*p65*-floxed) mouse, in which exons 7 and 10 were flanked by loxP sites, was previously described⁴¹. Mice harboring the *p65^{fl/fl}* alleles were crossed with mice expressing Cre recombinase under control of the cardiac-specific *Nkx2.5* promoter⁴². All experiments involving animals were approved by the Institutional Animal Care and Use Committees of the University of Washington and all studies were carried out in accordance with the approved guidelines.

For echocardiography, mice were anesthetized with 2% isoflurane by inhalation and scanning was performed with a VisualSonics Vevo 2100 imaging system as described previously⁴³. M-mode ventricular dimensions were averaged from 3–5 cycles. Fractional

shortening (FS) was calculated using ventricular dimensions in end of systole and diastole (LVES and LVED, respectively): $FS = [(LVED - LVES)/LVED] \times 100 (\%)$.

Surgical procedures of ischemia-reperfusion (I/R) injury and myocardial infarction (MI) in the mouse have been described previously⁴⁴. Briefly, a suture was tied with a slip-knot around the left anterior descending (LAD) coronary artery, and mice were revived by removal from anesthetic during 60 min of ischemia, after which the knot was released and the heart was reperfused for 24 h. The MI procedure was similar to I/R except that the ligature was permanent with no reperfusion. At the end of reperfusion, the LAD artery was re-occluded and 2% Evans blue dye was injected into the left ventricle to stain the normally perfused region blue and outline the area at risk (AAR). The heart was rapidly excised and cut into transverse sections and incubated in 2% 2,3,5- triphenyltetrazolium chloride (TTC) for 10 min at 37°C to differentiate the viable myocardium and infarcted area (IA). The areas of infarction, at risk, and nonischemic area were assessed using Image J (NIH).

Histological analysis

For histological analysis, adult hearts were fixed in 10% formalin/phosphate-buffered saline and dehydrated for paraffin embedding. Fibrosis was detected with Masson's Trichrome staining on 5- μ m paraffin sections. Blue collagen staining was quantified using Metamorph software³⁶. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in paraffin sections was performed with an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions⁴⁰. Immunoreactivity or TUNEL positivity was quantified from at least 20 random fields by light microscopy. Mouse cTnI plasma levels were measured using an ELISA kit from Life Diagnostics, Inc (West Chester, PA). Absorbance at 450 nm (sample) and 630 nm (reference) were measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT).

NF κ B activity assay.—NF κ B activation was measured using an enzyme-linked immunosorbent assay (ELISA)-based method (Trans-AM NF κ B; Active Motif, Carlsbad, CA) according to the manufacturer's guidelines. Briefly, cardiac lysates were placed in 96-well plates coated with an oligonucleotide containing the NF κ B consensus sequence. The presence of active NF κ B was detected by using a primary antibody specific for the p65 subunit bound to the consensus sequence, followed by incubation with a horseradish peroxidase-conjugated secondary antibody, colorimetric reaction, and absorbance measurements (450 nm).

Detection of ROS in the heart.—Using frozen cardiac sections, cellular production of ROS was detected with chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Invitrogen) staining following the manufacturer's instruction. Dihydroethidium (DHE) fluorescence analysis was also performed to examine the generation of superoxide. In brief, frozen sections (5 μ m) were prepared from mouse hearts and incubated with 10 μ M CMH₂DCFDA or DHE (Invitrogen) at 37°C for 30 min in the dark and washed three times with PBS. Fluorescence was visualized by confocal microscopy and quantified using ImageJ software.

Statistics

Comparisons between experimental groups were made using one-way ANOVA with Bonferroni post hoc test or repeated-measures ANOVA for multiple comparisons using the statistics module of GraphPad Prism 5.0 (GraphPad, San Diego, CA). Data are expressed as means $\pm$ SEM. A *P*-value of <0.05 was considered significant.

Results

NF κ B promotes oxidative stress-induced necrotic cell death

Although the anti-cell death function of NF κ B in response to a range of pathological signals has been well-established, its role in oxidative stress-induced cell death signaling remains elusive. Here, we determined whether and how NF κ B regulates cell death in the setting of oxidative stress, with a focus on the major transacting subunit NF κ B-p65. H₂O₂ and tBHP (tert-Butyl hydroperoxide) were used to generate oxidative stress, which has been shown to mainly induce non-apoptotic cell death⁴⁵. We found that H₂O₂ and tBHP markedly induced cell death in cardiomyocytes, as indicated by increased propidium iodide (PI) incorporation, an indicator of membrane disruption (Figure 1A,B). H₂O₂ and tBHP also promoted the release of HMGB1 (high mobility group box 1) into the culture supernatant, another marker of necrotic cell death^{37,46} (Figure 1C). Of note, our data showed that oxidative stress-induced necrosis was independent of RIP1/RIP3 necroptotic pathway (Supplemental Figure 1). Moreover, as an apoptotic marker, PARP cleavage was not detected under these conditions, whereas PAPR cleavage was clearly detected when stimulated with the classic apoptotic inducer TNF α and cycloheximide (CHX) (Figure 1C). These results indicate that oxidative stress induces necrotic cell death that is distinct from necroptosis or apoptosis. Importantly, ablation of NF κ B-p65 with an adenoviral vector encoding p65 shRNA (Ad-shp65) markedly inhibited H₂O₂- and tBHP-induced cell death as well as HMGB1 release (Figure 1A–C). Similar effects were observed using p65 $^{-/-}$ MEFs, further confirming a pro-cell death role of NF κ B-p65 in response to oxidative stress (Figure 1D,E).

To further determine the role of NF κ B signaling pathway in oxidative stress-induced cell death, we examined the rates of cell death in cardiomyocytes transduced with adenoviral vectors encoding a non-degradable I κ B α mutant (I κ B α -S32/36A; Ad-I κ B α M) to block NF κ B activation³⁷. Cell death induced by tBHP was significantly inhibited by I κ B α M expression (Figure 1F,G), suggesting that blockade of the canonical NF κ B pathway confers cell death resistance to oxidative stress. Next, we examined whether activation of NF κ B could promote oxidative stress-induced cell death. Indeed, overexpression of IKK β , which induces canonical NF κ B activation³⁹, further increased tBHP-induced cell death and HMGB1 release in cardiomyocytes (Figure 1F,G). Together, these results identified a pro-cell death role of NF κ B in oxidative stress induced necrosis, possibly through the canonical NF κ B signaling pathway.

NF κ B mediates cell death induced by simulated ischemia/reperfusion or doxorubicin

To further investigate the role of NF κ B in pathological conditions associated with elevated oxidative stress^{47–49}, cardiomyocytes were subjected to simulated ischemia and reperfusion (sI/R) or the chemotherapeutic agent doxorubicin (DOX). Intriguingly, NF κ B transcriptional

activity, as assessed by NF κ B luciferase reporter assay, was markedly elevated in cardiomyocyte after sI/R or DOX (Figure 2A). To examine the role of NF κ B in sI/R or DOX induced cell death, cardiomyocytes were transduced with Ad-shp65 or Ad-I κ B α M to block NF κ B activation (Figure 2B). The results showed that inhibition of NF κ B attenuated cell death induced by sI/R, which was associated with diminished HMGB1 release (Figure 2C,D). Similar effects were observed in cells treated with DOX (Figure 2E,F). These results further showed a pro-cell death role for NF κ B in pathological conditions involving oxidative stress.

NF κ B antagonizes the NRF2-ARE antioxidant pathway

The transcription factor Nrf2 (Nuclear factor erythroid 2-related factor 2) is a master regulator of cellular redox homeostasis, which binds to a *cis*-acting element ARE in the promoters of genes that encode antioxidant proteins⁵⁰. We found that overexpression of Nrf2 in cardiomyocytes was sufficient to inhibit cell death induced by tBHP, whereas ablation of Nrf2 further enhanced cell death, indicating a key role of Nrf2 in oxidative stress-induced cell death (Supplemental Figure 2). We then tested the hypothesis that NF κ B may crosstalk with the Nrf2-ARE pathway to regulate oxidative stress-induced cell death. We first examined whether NF κ B could affect Nrf2-ARE transcriptional activity in cardiomyocytes transduced with an ARE-luciferase reporter. As expected, ablation of p65 with Ad-shp65 largely inhibited basal NF κ B-luciferase activity in cardiomyocytes (Figure 3A,B). Surprisingly, ARE-luciferase activity was markedly enhanced with p65 deletion (Figure 3A,B). Conversely, overexpression of IKK β , which greatly induced NF κ B-luciferase activity, inhibited ARE-luciferase activity (Figure 3A,B). To further determine the interaction between these two pathways, we found that ARE-luciferase activity was markedly enhanced by Nrf2 overexpression, which was largely inhibited by IKK β overexpression (Figure 3C). Consistent with this observation, the expression of two Nrf2 target genes, NQO1 and HO-1, was also inhibited by IKK β under basal conditions as well as after Nrf2 overexpression (Figure 3D). Conversely, deletion of p65 increased ARE-luciferase activity under basal conditions as well as in response to Nrf2 overexpression (Figure 3E). Deletion of p65 also potentiated NQO-1 and HO-1 expression (Figure 3F). These results indicate that NF κ B antagonizes Nrf2-ARE antioxidant signaling pathway.

NF κ B-p65 coordinates with HDAC3 to suppress NRF2-ARE activity

To begin to assess the underlying mechanisms by which NF κ B represses Nrf2-ARE, we hypothesize that NF κ B may recruit corepressors, such as histone deacetylases, to inhibit Nrf2 regulated gene expression. Indeed, our immunoprecipitation experiments showed that p65 interacted with both Nrf2 and HDAC3 in cardiomyocytes (Figure 4A,B). Intriguingly, an interaction between Nrf2 and HDAC3 was also detected, which was abrogated upon p65 deletion, but further enhanced by p65 overexpression (Figure 4C). These results indicate that p65 is required to mediate the interaction between HDAC3 and Nrf2.

The findings above also suggest that HDAC3 might be involved in the transcriptional repression of Nrf2 by NF κ B p65. We then tested whether NF κ B signals through HDAC3 to repress the Nrf2-ARE pathway. Transfection with HDAC3 in cardiomyocytes reduced basal ARE-luciferase activity by ~ 4 fold compared with the vector control, indicating that

HDAC3 acts as a transcriptional repressor in Nrf2-ARE signaling (Figure 5A). Intriguingly, the repressing effect of HDAC3 was lost upon p65 deletion in cardiomyocytes (Figure 5B). Moreover, ablation of p65 with Ad-shp65 increase basal ARE-luciferase activity, and overexpression of HDAC3 had no effect on ARE-luciferase activity in these p65-deficient cells, although an inhibitory effect of HDAC3 was detected in the control cells (Figure 5B). These data suggest that p65 is required for the inhibitory action of HDAC3 on Nrf2-ARE transcriptional activity, a finding consistent with our observation that p65 mediates the interaction between HDAC3 and Nrf2 (Figure 4C).

To further determine the involvement of HDAC proteins in NF κ B mediated repression of Nrf2-ARE activity, we assess the inhibitory effect of p65 on ARE-Luc activity in the presence and absence of trichostatin A (TSA, 10 nM), a HDACs inhibitor. As expected, overexpression of p65 reduced basal ARE-luciferase activity by ~ 50% (Figure 5C). TSA induced a 3.5-fold increase in basal ARE-luciferase activity (Figure 5C). Moreover, the inhibitory effect of p65 on ARE-luciferase was also blocked in the presence of TSA (Figure 5C). These results further suggest that NF κ B p65 mediated transcriptional silencing of Nrf2-ARE requires the deacetylase activity of HDAC proteins.

Based on our observation that both p65 and HDAC3 are involved in transcriptional repression of Nrf2-ARE, we reasoned that inhibition of HDAC or NF κ B would inhibit oxidative stress-induced cell death by promoting Nrf2-mediated antioxidative signaling. The functional effects of inhibiting HDAC or NF κ B were examined in cardiomyocytes subjected to sI/R. As shown in Figure 5D, inhibition of HDACs or NF κ B with TSA and BMS-345541, respectively, diminished cell death induced by sI/R, suggesting both NF κ B and HDAC3 are functionally involved in this process.

Ablation of p65 attenuates cardiac ischemic injury and pathological remodeling *in vivo*.

—It has been shown that NF κ B, especially its major subunit p65, was markedly upregulated in the myocardium after I/R injury^{27–29}. We assessed NF κ B activity in ventricular lysates using a TransAM NF κ B p65 assay. NF κ B activity was not changed during ischemia, but quickly increased in the heart at 5 min and at 60 min of reperfusion; this increase was sustained throughout 24 h of reperfusion (Supplemental Figure 3). The dynamic changes in myocardial NF κ B activity correlate with ROS production as assessed by chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) and dihydroethidium (DHE) fluorescence assays (Supplemental Figure 3). We hypothesize that NF κ B plays a pathogenic role in cardiac ischemic/reperfusion injury *in vivo* by promoting myocardial cell death. To test this hypothesis, we employed a cardiac-specific *p65* knockout mice as we previously described³⁹, by crossing mouse homozygous for the *p65-loxP*-targeted allele (*p65^{fl/fl}*) with *Nkx2.5-Cre* “knock-in” mouse. Western blotting of protein extracts from hearts of 2-month old *p65^{fl/fl}*Nkx2.5-Cre mice showed >80% reduction in p65 protein compared with *p65^{fl/fl}* control mice (Figure 6A). As we previously reported, *p65^{fl/fl}*Nkx2.5-Cre mice were generated at predicted mendelian ratios and showed no discernable phenotype at baseline³⁹.

To determine the functional effects of p65 deletion in the heart *in vivo*, *p65^{fl/fl}*Nkx2.5-Cre and *p65^{fl/fl}* control mice were subjected to I/R with left anterior descending coronary artery

ligation for 1 h followed by reperfusion for 24 h. As shown in Figure 6B, deletion of p65 significantly reduced NF κ B activity by >60% compared with *p65^{fl/fl}* control mice at baseline (Figure 6B; sham groups). A ~2 fold increase in cardiac NF κ B activity was induced by I/R, which was also largely abrogated in *p65* deleted hearts (Figure 6B; I/R groups). The effect of p65 ablation on cardiac susceptibility to I/R injury was then assessed. Infarct size after I/R was significantly reduced in *p65* knockout hearts (infarct size/area at risk: $39.7 \pm 3.2\%$ in *p65^{fl/fl}* versus $24.2 \pm 4.5\%$ in *p65^{fl/fl}Nkx2.5-Cre*; $P < 0.05$; Figure 6C). The area at risk was not different in *p65* knockout and control littermates (area at risk: $48.3 \pm 2.3\%$ in *p65^{fl/fl}* versus $50.1 \pm 2.9\%$ in *p65^{fl/fl}Nkx2.5-Cre*; $P > 0.05$). Given that cardiomyocyte cell death plays a key role in cardiac I/R injury, the extent of cell death was examined at 24 h reperfusion after 1 h ischemia. A significant decrease in TUNEL positive cells was detected in *p65* knockout mice compared with control mice (Figure 6D). Of note, TUNEL analysis detects both apoptotic and necrotic cell death *in vivo*^{51,52}. *p65* knockout mice also displayed reduced plasma levels of cTnI (cardiac troponin I), a biomarker of necrotic cell death and myocardial damage⁵³ (Figure 6E). These results indicate that genetic ablation of p65 in the heart prevented cardiac cell death and ischemic injury *in vivo*.

Lastly, we examined the role of NF κ B p65 in pathological remodeling and functional recovery after chronic myocardial infarction (MI). *p65^{fl/fl}Nkx2.5-Cre* and *p65^{fl/fl}* control mice were subjected to permanent ligation of left anterior descending coronary artery or a sham procedure for 2 weeks. Masson's trichrome staining of cardiac sections showed less extensive scar tissue and less ventricular dilation in *p65^{fl/fl}Nkx2.5-Cre* mice after 2 weeks of MI compared with *p65^{fl/fl}* control mice (Figure 7A). Of note, there was no significant difference in initial infarct size as measured 24 h after permanent MI (initial infarct at 24 h: $43.8 \pm 2.1\%$ in *p65^{fl/fl}* versus $41.4 \pm 2.2\%$ in *p65^{fl/fl}Nkx2.5-Cre*; $P > 0.05$). Intriguingly, *p65* knockout mice displayed less secondary hypertrophy following 2 weeks of MI, as indicated by decreased heart weight and body weight ratio (Figure 7B). To assess the functional recovery after MI, echocardiographic analysis of ventricular chamber dimensions and fractional shortening showed better preserved cardiac contractile function with less ventricular chamber dilation in *p65* knockout mice (Figure 7C–E). Taken together, these results indicate that ablation of p65 in the heart improved pathological remodeling and functional recovery after chronic myocardial infarction, suggesting a new pathogenic role of NF κ B, and thus a potential therapeutic target, in cardiac ischemic injury and pathological remodeling.

Discussion

In this study, we identified a new role for NF κ B p65 in promoting necrotic cell death in the setting of oxidative stress. In contrast to the well-defined pro-survival function of the NF κ B pathway, the pro-cell death role of NF κ B and the biological relevance in disease pathogenesis remain largely undefined, and the underlying mechanisms have not been investigated. Our data provide novel insight into the new pro-cell death role of NF κ B through transcriptional repression of the Nrf2-ARE antioxidative pathway. Furthermore, our results provide new mechanistic evidence that the Nrf2-ARE pathway is transcriptionally repressed by the cooperative actions of NF κ B p65 and HDAC3. Using a cardiomyocyte-specific NF κ B p65 KO model, our studies revealed key maladaptive effects of NF κ B p65

within the cardiomyocyte compartment in promoting cell death, I/R injury, and pathological remodeling *in vivo*. These results identified a new pathogenic role of NFκB in oxidative stress induced necrosis and cardiac ischemic injury, suggesting that inhibition of key regulators of this pathway, such as p65 and HDAC3, may provide therapeutic benefit in cardiac I/R injury and other disease conditions associated with oxidative stress.

Our results showed that NFκB plays a critical role in mediating necrotic cell death in response to oxidative stress, which is distinct from apoptosis or necroptosis. Importantly, this effect is mediated by the p65 subunit, which negatively regulates the Nrf2-ARE anti-oxidative pathway via transcriptional silencing. Consistent with our data, Ho et al. reported that NFκB potentiated hydrogen peroxide induced cell death in MEFs²⁶. The detailed mechanism was unclear, but this effect was associated with the repression of pro-survival genes and induction of pro-death genes. Similarly, a pro-cell death role of NFκB has also been reported in cardiomyocytes^{54,55}. Therefore, NFκB serves as a control point that can induce either survival or death depending on the cell type and nature of the stimulus⁵⁶. We speculate that the mechanism of NFκB activation could profoundly influence its nuclear function. Classic activators of NFκB, such as inflammatory cytokines and bacterial products, induced rapid NFκB nuclear translocation that peaks within 1 hour. In contrast, “atypical” activators of NFκB such as oxidative stress, UV, and chemotherapeutic compounds, induced NFκB DNA binding through a distinct pathway with slower kinetics^{26,57}. This atypical pathway includes an IKK independent mechanism and Tyr42 phosphorylation of IκBα^{9,58}. In addition, p65 phosphorylation is differentially regulated by atypical NFκB activators²⁵, which may regulate differential phosphorylation of RelA (p65), affecting its ability to interact with coactivators and corepressors, such as HDACs⁵⁹.

Our finding that NFκB inhibits the Nrf2-ARE antioxidant pathway by transcriptional silencing highlights a less defined but emerging role for NFκB as a transcriptional repressor^{12,25,60,61}, despite NFκB’s well-established function as a transcriptional activator. This raises the interesting possibility that NFκB may promote oxidative stress-induced cell death by actively repressing pro-survival genes such as Nrf2. Consistent with this notion, we found that deletion of Nrf2 markedly increased cell death induced by oxidative stress, whereas overexpression of Nrf2 showed the opposite effect by inhibiting cell death. The transcription factor Nrf2 is essential for cellular defense against oxidative stress through transcriptional activation of antioxidant responsive element (ARE)-mediated antioxidant gene expression, such as NQO1, HO-1, NAD(P)H, GPX, SOD, catalase, and thioredoxin⁶².

It has been shown that Nrf2 is acetylated within its Neh1 DNA-binding domain by p300/CBP, which increases its DNA-binding capacity and downstream gene expression⁶³. Moreover, Nrf2 acetylation is enhanced by the HDAC inhibitor TSA⁶⁴, suggesting HDAC(s) may negatively regulate Nrf2 activation. Consistent with our finding that NFκB suppresses the Nrf2-ARE pathway in cardiomyocytes, Yu et al. showed that NFκB p65 inhibited Nrf2 transcriptional activity by interacting with Keap1 and promoting its nuclear translocation in HEK293 cells⁶⁵. Moreover, it was reported that NFκB p65 inhibited Nrf2-ARE signaling by depriving p300/CBP from Nrf2 in HepG2 cells⁶⁶. Here we provide new evidence that NFκB p65 inhibits the Nrf2-ARE pathway by recruiting HDAC3 in cardiomyocytes. It is possible that distinct mechanisms may exist to regulate NFκB mediated suppression of the Nrf2-ARE

antioxidant pathway depending on cell types and cellular contexts. Nonetheless, our results are in agreement with previous observations that NFκB p65 antagonizes the Nrf2-ARE pathway^{65,66}. Our study further expands these findings by revealing the biological implications of this NFκB-Nrf2 crosstalk mechanism in oxidative stress-induced necrosis, which has not been explored in the previous studies. Indeed, transcriptional inhibition of Nrf2 provides a new mechanism by which NFκB promotes oxidative stress induced necrotic cell death. Whether NFκB signals through other pathways to regulate pro-survival or pro-cell death gene expression is an area of active investigation.

Our immunoprecipitation experiments revealed that NFκB p65 interacts with HDAC3 in cardiomyocytes. Furthermore, we found that the inhibitory actions of NFκB p65 on Nrf2 transcriptional activity was alleviated by the HDAC inhibitor TSA, whereas HDAC3 overexpression markedly inhibited Nrf2-ARE activity. These observations strongly suggest the involvement of deacetylase activity in NFκB mediated repression of Nrf2 transcription. We could not exclude the possibility that other HDAC isoform(s) may play a redundant role in transcriptional inhibition of Nrf2, which needs to be further investigated. The interrelationship between HDAC3 and NFκB p65 is further demonstrated by our finding that functional inactivation of either factor was sufficient to promote Nrf2 transcriptional activity and inhibit oxidative stress-induced cell death. Moreover, deletion of NFκB p65 not only abrogated HDAC3-Nrf2 interaction but also blocked the inhibitory actions of HDAC3 on Nrf2 transcription. These findings are consistent with a model in which NFκB p65 serves as a platform for the recruitment of HDAC activity to Nrf2-ARE. The finding that HDAC proteins suppresses Nrf2 activation is consistent with a previous study showing that the HDAC inhibitor TSA promotes Nrf2 activation and downstream gene expression by increasing Nrf2 acetylation, although the HDAC isoform(s) involved was unclear⁶⁴. It was suggested that the recruitment of HDAC activity may help maintain local chromosome in a condensed “histone hypoacetylation” state and hence impede local gene transcription. Given that HDACs can also regulate gene transcription by modifying nonhistone DNA binding factors such as transcription factors^{12,16–18}, we propose that HDAC3 may directly suppress Nrf2 activity via deacetylation through a cooperative action of NFκB p65.

This study also revealed a new pathogenic role of NFκB in mediating cardiomyocyte death and cardiac I/R injury. p65 is the major NFκB subunit that is activated in the heart following myocardial infarction⁵⁵. Using a cardiac-specific *p65* KO mouse model, we showed that genetic deletion of p65 within the cardiomyocyte largely inhibited the ability of I/R to increase NFκB activation. Moreover, p65 gene deletion attenuated I/R-induced cardiomyocyte death, infarct development, and contractile dysfunction. Consistent with our results, Zhang et al. observed a similar cardioprotective effect in another cardiomyocyte-specific p65 knockout mouse model with *p65^{fl/fl}* x *αMHC-Cre*³³. In that study, the cardioprotective effect of p65 deletion was associated with enhanced calcium uptake by the SR and improved calcium handling, but the mechanism by which p65 regulates cell death and I/R injury remained unclear³³. In addition to its role in acute I/R injury, our results demonstrate that averting cardiomyocyte autonomous activation of NFκB by p65 deletion also significantly attenuated chronic cardiac remodeling and dysfunction after myocardial infarction. These results indicate that the NFκB within the cardiomyocytes, especially the p65 subunit, plays a critical role in the pathogenesis of cardiac ischemic injury and

pathological remodeling. Our data provide evidence that ablation of p65 protects the heart from I/R injury, at least in part, by inhibiting cardiomyocyte death. Of note, it has been reported that inactivation of NF κ B reduced proinflammatory cytokine expression and diminished myocardial inflammatory response^{33,55}, which may also contribute to improved remodeling and functional recovery after chronic myocardial infarction.

In summary, our results identified an unexpected role of NF κ B in promoting oxidative stress-induced necrosis in cardiomyocytes and mouse embryonic fibroblasts. We provide mechanistic evidence that the pro-cell death role of NF κ B in response to oxidative stress involves active transcriptional repression of NRF2-ARE through cooperative actions of deacetylase activity of HDAC3. We further demonstrate that genetic ablation of NF κ B-p65 attenuated oxidative stress-induced necrotic cell death *in vitro* and cardiac I/R injury *in vivo*, revealing a new pathogenic role of NF κ B in cardiac ischemic injury and remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Inactivation of NF κ B inhibited, whereas activation of NF κ B promoted, oxidative stress-induced necrosis.
- NF κ B negatively regulated Nrf2-ARE activity and the expression of antioxidant proteins.
- NF κ B-p65 is required for Nrf2-HDAC3 interaction and transcriptional silencing of Nrf2-ARE activity.
- Inhibition of NF κ B or HDACs increased Nrf2-ARE activity and promoted cell survival after simulated ischemia/reperfusion.
- Genetic ablation of NF κ B-p65 in the mouse heart attenuated ischemia/reperfusion injury and improved cardiac remodeling and functional recovery.

Figure 1. Ablation of p65 inhibits oxidative stress-induced necrosis.

A. Cell death assessed by propidium iodide (red) and Hoechst 33342 (blue) staining of cardiomyocytes infected with Ad-shp65 (adenovirus encoding p65 shRNA) or Ad-shScram (scrambled control adenovirus), followed by treatment with tBHP, H₂O₂, or vehicle control for 12 h. **B.** Quantification of cell death (% PI positive) from cells treated as in A. **P* < 0.05 vs Control; #*P* < 0.05 vs Ad-βgal. n = 4. **C.** Western blots for the indicated proteins in culture supernatant (sup.) and whole cell extracts from cardiomyocytes treated as in A. HMGB1 released into culture supernatant is a biomarker for necrosis. * indicates cleaved PARP, a marker of apoptosis. **D.** Quantification of cell death from *p65*^{+/+} and *p65*^{-/-} MEFs treated with tBHP, H₂O₂, or vehicle control for 12 h. **P* < 0.05 vs Control; #*P* < 0.05 vs *p65*^{+/+}. n = 4. **E.** Western blots for the indicated proteins from cells treated as in D. **F.** Quantification of cell death from cardiomyocytes infected with Ad-βgal (β-galactosidase), Ad-IκBαM (IκBα-S32/36A mutant), or Ad-IKKβ, followed by tBHP treatment for 12 h. **P* < 0.05 vs Control;

$P < 0.05$ vs Ad- β gal tBHP. n = 4. **G.** Western blots for the indicated proteins from cells treated as in F.

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Figure 2. Inhibition of NFκB reduces simulated ischemia/reperfusion (sI/R) or doxorubicin (DOX) induced cell death in cardiomyocytes.

A. NFκB-luciferase activity in cardiomyocytes infected with recombinant adenoviruses expressing NFκB-luciferase reporter and subjected to 4 h simulated ischemia and 12 h reperfusion (sI/R) or control condition (left panel). NFκB-luciferase activity was also measured in cells treated with DOX (10 μM for 8 h) or vehicle control (right panel). * $P < 0.05$ vs Control or Vehicle. $n = 4$. **B.** Western blot for the indicated proteins from cardiomyocytes infected with Ad-βgal, Ad-shp65 (p53 shRNA adenovirus), or Ad-IκBαM (IκBα-S32/36A mutant) for 24 h. **C.** Quantification of cell death from cardiomyocytes infected with Ad-βgal, Ad-shp65, or Ad-IκBαM and subjected to sI/R or control condition. * $P < 0.05$ vs Control; # $P < 0.05$ vs Ad-βgal. $n = 4$. **D.** Western blots for the indicated proteins from cells treated as in C. **E.** Quantification of cell death from cardiomyocytes infected with Ad-βgal, Ad-shp65, or Ad-IκBαM, followed by treatment with DOX or vehicle control for 12 h. * $P < 0.05$ vs Vehicle; # $P < 0.05$ vs Ad-βgal. $n = 4$. **F.** Western blots for the indicated proteins from cells treated as in E.

Figure 3. NFκB negatively regulates the NRF2-ARE antioxidant pathway.

A. Western blots for the indicated proteins from cardiomyocytes infected with Ad-βgal, Ad-shp65, or Ad-IKKβ for 24 h. **B.** NFκB-luciferase activity in cardiomyocytes infected with recombinant adenoviruses expressing NFκB-luciferase reporter along with Ad-βgal, Ad-shp65, or Ad-IKKβ for 24 h (left panel). ARE-luciferase activity in cardiomyocytes transfected with an ARE-luciferase reporter followed by infection with Ad-βgal, Ad-shp65, or Ad-IKKβ for 24 h (right panel). * $P < 0.05$ vs Ad-βgal. # $P < 0.05$ vs Ad-βgal or Ad-shp65. $n = 4$. **C.** ARE-luciferase activity in cardiomyocytes transfected with an ARE-luciferase reporter followed by infection with adenoviruses encoding the indicated proteins. * $P < 0.05$ vs Ad-βgal; # $P < 0.05$ vs Ad-Nrf2. $n = 4$. **D.** Western blots for the indicated proteins from cells treated as in C. **E.** ARE-luciferase activity in cardiomyocytes transfected with an ARE-luciferase reporter followed by infection with the indicated adenoviral vectors. * $P < 0.05$ vs Ad-βgal; # $P < 0.05$ vs Ad-Nrf2. $n = 4$. **F.** Western blots for the indicated proteins from cells treated as in E.

Figure 4. p65 mediates Nrf2-HDAC3 interaction.

A. Western blots for Nrf2 and p65 after immunoprecipitation (IP) with anti-p65 antibody or preimmune IgG from cellular extracts of cardiomyocytes. **B.** Western blots for HDAC3 and p65 after immunoprecipitation with anti-p65 antibody or preimmune IgG. **C.** Western blots for HDAC3 and Nrf2 after immunoprecipitation with anti-Nrf2 antibody from cardiomyocytes infected with Ad-βgal, Ad-shp65, or Ad-p65. **D.** Western blots for the indicated proteins in cellular lysate (input) from cells treated as in C.

Figure 5. p65 is required for HDAC3-mediated inhibition of NRF2-ARE activity, and inhibiting HDACs or NFκB reduces si/R-induced cardiomyocyte death.

A. ARE-luciferase activity in cardiomyocytes transfected with an ARE-luciferase reporter followed by infection with Ad-βgal or Ad-HDAC3. * $P < 0.05$ vs Ad-βgal. $n = 3$. **B.** ARE-luciferase activity in cardiomyocytes transfected with an ARE-luciferase reporter followed by infection with the indicated adenoviral vectors. * $P < 0.05$ vs Ad-βgal; # $P < 0.05$ vs Ad-shScram. $n = 3$. n.s., non-significant. **C.** ARE-luciferase activity in cardiomyocytes transfected with an ARE-luciferase reporter and infected with Ad-βgal or Ad-p65, followed by treatment with TSA (Trichostatin A; HDAC inhibitor) or vehicle control. * $P < 0.05$ vs Ad-βgal; # $P < 0.05$ vs Control. $n = 3$. n.s., non-significant. **D.** Quantification of cell death from cardiomyocytes pretreated with TSA, BMS-345541 (IKK inhibitor), or vehicle before subjecting to si/R or control condition. * $P < 0.05$ vs Control; # $P < 0.05$ vs Vehicle si/R. $n = 4$ per group.

Figure 6. Cardiomyocyte-specific p53 knockout attenuates cardiac ischemia/reperfusion injury *in vivo*.

A. Western blot for p53 and α-tubulin in heart extracts from *p53^{fl/fl}* and *p53^{fl/fl}Nkx2.5Cre* mice. **B.** Measurement of cardiac NF-κB-p53 activity by TransAM oligonucleotide binding assay in *p53^{fl/fl}* and *p53^{fl/fl}Nkx2.5Cre* mice subjected to I/R (60 min/24 h) or sham surgery. **P* < 0.05 vs *p53^{fl/fl}* Sham; †*P* < 0.05 vs *p53^{fl/fl}* Sham; #*P* < 0.05 vs *p53^{fl/fl}* I/R. n = 4 per group. **C.** TTC staining for cardiac infarct area in *p53^{fl/fl}* and *p53^{fl/fl}Nkx2.5Cre* mice after I/R. Infarct area (IA) was normalized by area at risk (AAR). **P* < 0.05 vs *p53^{fl/fl}*. n = 5 per group. **D.** TUNEL analysis from hearts of *p53^{fl/fl}* and *p53^{fl/fl}Nkx2.5Cre* mice after I/R. **P* < 0.05 vs *p53^{fl/fl}*. n = 5 per group. **E.** Plasma cTnI levels in *p53^{fl/fl}* and *p53^{fl/fl}Nkx2.5Cre* mice after I/R or sham surgery. **P* < 0.001 vs Sham; #*P* < 0.05 vs *p53^{fl/fl}* I/R. n = 5 per group.

Figure 7. Cardiomyocyte-specific p65 knockout improves cardiac remodeling and functional recovery after chronic myocardial infarction.

A. Masson's trichrome-stained cardiac sections and quantitation of infarct size from *p65^{fl/fl}* and *p65^{fl/fl}Nkx2.5Cre* mice subjected to MI (myocardial infarction) or a sham procedure for 2 weeks. The scar size was calculated as the average of middle ring slices and expressed as percentage of left ventricle. **P* < 0.05 vs *p65^{fl/fl}*. *n* = 5 per group. **B.** Heart weight normalized by body weight (HW/BW ratio) from mice indicated in A. **P* < 0.05 vs Sham; #*P* < 0.05 vs *p65^{fl/fl}* MI. *n* = 5 per group. **C.** Representative echocardiographic M-mode images from *p65^{fl/fl}* and *p65^{fl/fl}Nkx2.5Cre* mice subjected to MI for 2 weeks. The vertical white lines indicate left ventricular end-diastolic dimension (LVED). **D** and **E.** Echocardiographic measurement of left ventricular end-diastolic dimension (LVED) and fractional shortening (FS) after 2-week MI. **P* < 0.05 vs Sham; #*P* < 0.05 vs *p65^{fl/fl}* MI. *n* = 5 per group.