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NF- κ B inhibition compromises cardiac fibroblast viability under hypoxia

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Abstract

Cardiac fibroblasts are reported to be relatively resistant to stress stimuli compared to cardiac myocytes and fibroblasts of non-cardiac origin. However, the mechanisms that facilitate their survival under conditions of stress remain unclear. We explored the possibility that NF- κ B protects cardiac fibroblasts from hypoxia-induced cell death. Further, we examined the expression of the anti-apoptotic cIAP-2 and Bcl-2 in hypoxic cardiac fibroblasts, and their possible regulation by NF- κ B. Phase contrast microscopy and propidium iodide staining revealed that cardiac fibroblasts are more resistant than pulmonary fibroblasts to hypoxia. Electrophoretic Mobility Shift Assay showed that hypoxia activates NF- κ B in cardiac fibroblasts. Supershift assay indicated that the active NF- κ B complex is a p65/p50 heterodimer. An I- κ B-super-repressor was constructed that prevented NF- κ B activation and compromised cell viability under hypoxic but not normoxic conditions. Similar results were obtained with Bay 11-7085, an inhibitor of NF- κ B. Western blot analysis showed constitutive levels of Bcl-2 and hypoxic induction of cIAP-2 in these cells. NF- κ B inhibition reduced cIAP-2 but not Bcl-2 levels in hypoxic cardiac fibroblasts. The results show for the first time that NF- κ B is an important effector of survival in cardiac fibroblasts under hypoxic stress and that regulation of cIAP-2 expression may contribute to its pro-survival role.

Introduction

Cardiac fibroblasts, the most abundant cell type in the heart, are a major source of myocardial collagen, matrix metalloproteinases and several critical growth factors and cytokines [1, 2]. They are primarily involved in wound healing following injury, and there is increasing appreciation of their roles in maintaining the structural and functional integrity of the myocardium [3]. Unlike cardiac myocytes that have limited replicative capacity after birth, cardiac fibroblasts retain their proliferative potential throughout adult life, which is central to their role in tissue repair. In a setting of myocardial injury, quiescent interstitial fibroblasts undergo phenotypic transformation into myofibroblasts that infiltrate the site of injury, proliferate, and produce matrix components, resulting in scar formation. This implies that cardiac fibroblasts are uniquely programmed to resist cell death and take part in the tissue response to injury under conditions that do not favour the survival of co-resident cells in the heart. Moreover, several *in vitro* studies show that cardiac fibroblasts are relatively resistant to a variety of pro-apoptotic stimuli [4]. In this regard, although apoptosis of myofibroblasts marks the termination of wound-healing responses in non-cardiac tissues,

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myofibroblasts in the heart are reported to persist in the infarct scar long after the healing phase is completed, without going through apoptosis [5]. Failure of normal apoptosis of myofibroblasts *in vivo* can potentially result in over-expression of the fibroproliferative response *post-injury* and contribute to inappropriate matrix deposition and pump dysfunction, leading to heart failure [3]. Barring a single report that ascribes a predominant role to constitutively expressed Bcl-2 in cardiac fibroblast survival under conditions of ambient stress [4], the molecular mechanisms underlying the relative resistance of cardiac fibroblasts to cell death remain unclear.

Hypoxia is a major stress condition influencing the extent of cell injury and death during acute and chronic myocardial ischemia and infarction [6]. It triggers mitochondrial permeability and apoptosis in cardiomyocytes [7, 8]. In fact, significant loss of cardiomyocytes by apoptosis is an important pathogenic feature of cardiac ischemia and infarction [9]. Hypoxia has also been shown to induce apoptosis in endothelial cells and vascular smooth muscle cells [10, 11]. On the contrary, cardiac fibroblasts are resistant to hypoxia-induced cell death [4]. Hypoxia is reported to activate NF- κ B [12], a stress-activated transcription factor that can exert both pro- and anti-apoptotic effects depending upon the cell type, extent of NF- κ B activation and nature of the apoptotic stimuli [13,14]. The major mechanism by which NF- κ B suppresses apoptosis involves transcriptional regulation of specific anti-apoptotic proteins such as cIAP-1, cIAP-2, xIAP and Bcl-2 [15]. In the heart, NF- κ B is activated in response to hypoxia [16] but different cell types are reported to respond differently to NF- κ B [17, 18]. Whether NF- κ B contributes to the resistance of cardiac fibroblasts to stress-induced cell death is unknown.

Using the I- κ B super-repressor (I- κ B-sr) to inhibit NF- κ B activation, we provide evidence here that NF- κ B plays a role in cardiac fibroblast survival under hypoxic conditions. Further, the findings raise the possibility that regulation of cIAP-2 expression by NF- κ B may contribute to its pro-survival role.

Materials and Methods

All fine chemicals for cell culture, fetal bovine serum (FBS), Bay 11-7085, caspase-3 assay kit and Hoechst 33342 were purchased from Sigma-Aldrich, USA. All cell culture ware was purchased from BD Falcon, USA. Primary antibodies against cIAP-2, Bcl-2, β -actin and NF- κ B subunits, and primers for NF- κ B were obtained from Santa Cruz Biotechnology, USA. Cell lysis buffer was from Cell Signaling Technologies, USA. Enhanced chemiluminescence reagent kit and the BCA protein assay kit were from Pierce, USA. The Qiagen Maxi prep kit was used for plasmid isolation. Restriction enzymes were procured from New England Bio Labs, USA. Basic Nucleofector kit for electroporation of primary mammalian fibroblasts was from Lonza, Swiss. NucBuster nuclear protein extraction kit was from Novagen, USA. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study had the approval of the Institutional Animal Ethics Committee (B29112005 VII).

Plasmid construction

I κ B-super repressor (I κ B-sr-EGFP) was constructed from plasmid pLL143 that carries the I κ B-sr gene with the internal ribosome entry site (IRES) and EGFP (reporter) sequences. *E.coli* DH5- α strain carrying pLL143 was maintained as an 8% glycerol stock in LB broth at -80°C . Plasmid pLL143 was isolated using Qiagen's Maxi prep kit as per manufacturer's instructions. The poly-A tail of the plasmid that masked the EGFP expression was spliced using BglII and EcoRI, the staggered ends were blunted, circularized and electrophoresed on a 1% low melting agarose gel for an hour. The new plasmid was approximately 6240 bp

and it retained all features of pLL143 but expressed EGFP in addition to I κ B-sr, unlike pLL143. The construction was validated by restriction mapping using restriction enzymes, BamH1 and XhoI, that yielded an 824 bp product in addition to the linear plasmid. Further validation was performed by western blotting using specific antibodies for I- κ B-sr and EGFP following standard protocols.

Isolation of fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague-Dawley rats (2–3 months) and characterized as described earlier [19]. Cells from passage 2 or 3 were used for the experiments. Pulmonary fibroblasts were isolated from the same animal source following essentially the same procedure as that for cardiac fibroblasts.

In vitro hypoxia

Hypoxic (pO₂ ~0.75 mmHg/5% CO₂) and normoxic (pO₂ ~150 mmHg/5% CO₂ “control”) conditions were generated using the BBL-GasPak system and GasPak envelopes from Becton Dickinson. The pO₂ of the hypoxic and normoxic media was ~23 and ~113 mmHg, respectively, and the pH was comparable. Cells were exposed to normoxic/hypoxic conditions, generally, for 12 hours, except where indicated otherwise.

Hoechst 33342/propidium iodide staining

Sub-confluent cultures (~50%) of cardiac or pulmonary fibroblasts in M199 with 10% FBS were subjected to the experimental conditions indicated and were incubated with Hoechst 33342 (10 μ M) at 37°C for 10 minutes. Following incubation in the dark, propidium iodide (PI) was added (0.25 μ g/ml) and cultures were visualized under a fluorescence microscope (Zeiss Axioskop 2 Plus) at excitation wavelengths of 352 nm and 538 nm, respectively, for Hoechst 33342 and PI.

Electrophoretic Mobility Shift Assay (EMSA)

DNA-binding activity of NF- κ B in cardiac fibroblasts was assessed by EMSA using standard protocol [20]. Confluent cardiac fibroblast cultures in M199 containing 0.1% FBS were subjected to hypoxic and/or normoxic treatments and nuclear extracts were prepared using the NucBuster Protein extraction kit. The nuclear fraction was aliquoted and stored at –80°C until use. Protein concentration of the nuclear extracts was determined using the BCA protein assay kit as per the manufacturer’s instructions. Primers for NF- κ B were labelled with γ ³²P ATP using T4 kinase. After the labelling reaction, the reaction mixtures were cleared with G-50 minicolumns to separate the labelled probes from free γ ³²P ATP. The nuclear extracts were incubated with the radiolabeled probes at room temperature for 90 minutes, and electrophoresed on a 6 % non-denaturing DNA retardation gel at 150V for 50 minutes. The gels were then processed for autoradiography.

Transfection of cardiac fibroblasts

Basic Nucleofector kit for primary mammalian fibroblasts was used for transfection, using the program recommended by the company (Program Number, U-23). I- κ B-sr-transfected cardiac fibroblasts in M199 with 10% FBS were subjected to 12 hours of hypoxia or normoxia and then stained with Hoechst 33342.

Bcl-2 and cIAP-2 expression in cardiac fibroblasts

Expression levels of Bcl-2 and cIAP-2 proteins were determined by western blot analysis as described earlier [21] with minor modifications. Briefly, 80%-90% confluent cultures of cardiac fibroblasts in M199 with 10% FBS exposed to hypoxic/normoxic treatments for the indicated durations were lysed in cell lysis buffer. Protein concentration was determined

using the BCA protein assay kit, as per the manufacturer's instructions. Following SDS-PAGE, the proteins were electro transferred to nitrocellulose membrane, which was then incubated with anti-cIAP-2 or anti-Bcl-2 antibodies. Immunoblots were then exposed to appropriate peroxide-conjugated secondary antibodies and developed using the enhanced chemiluminescent reagent kit. The membrane was stripped overnight on a rocking platform, re-probed with anti- β -actin primary antibody and developed as described above.

Caspase-3 activity assay

Caspase-3 assay was performed using commercial kits, following the manufacturer's instructions. Briefly, confluent cardiac fibroblast cultures in M199 with 10% FBS were exposed to hypoxic/normoxic treatments for the indicated durations, with or without Bay 11-7085 at 4 μ mol/L, and lysed in caspase lysis buffer. Cell lysates were incubated overnight with the substrate, Ac-DEVD-p-nitroaniline, at 37°C and the released p-nitroaniline was quantified spectrophotometrically at 405 nm. Caspase-3 activity was expressed in arbitrary units.

Statistics

Student's t-test was used to examine differences between experimental groups. Significance was determined at $p \leq 0.05$.

Results

Cardiac fibroblasts are resistant to hypoxia

To test whether cardiac fibroblasts are specifically resistant to hypoxia, cardiac and pulmonary fibroblast cultures were exposed to hypoxic or normoxic conditions, and cell survival was evaluated by phase contrast microscopy and Hoechst/PI staining. As our previous studies have shown that cardiac fibroblasts remain viable even at 48 hours of hypoxia, we examined whether pulmonary fibroblasts would also withstand 48 hours of hypoxia. In contrast to cardiac fibroblasts that exhibited normal morphology at 48 hours of hypoxia treatment (Fig. 1A), exposure of pulmonary fibroblasts to hypoxia for 48 hours resulted in extensive damage, as evidenced by phase contrast microscopy (Fig. 1B). Consistent with these observations, cardiac fibroblasts were hardly positive for PI (Fig. 1C) whereas a significant increase in the number of PI-stained cells was observed in pulmonary fibroblast cultures upon shorter exposure to hypoxia (Fig. 1D), showing that cardiac fibroblasts are uniquely programmed to resist hypoxic injury.

NF- κ B is activated in cardiac fibroblasts during hypoxic treatment

To ascertain whether NF- κ B plays a pro-survival role in cardiac fibroblasts during hypoxia, its activation status was first assessed by EMSA using nuclear extracts from cardiac fibroblasts. The duration of exposure of cells to normoxic or hypoxic conditions was 6 hours in these experiments as activation of NF- κ B is an early response to stress. Moreover, since preliminary experiments showed that 10% fetal calf serum per se activates NF- κ B, 0.1% serum was used in this experiment subsequently. As shown in Fig. 2A, hypoxia induced NF- κ B (lane H). The binding of nuclear extracts to the probe appeared to be NF- κ B-specific as non-radiolabeled probes competed off the binding (Fig. 2B, lane Hw), and a single-base mutation within NF- κ B binding sequence abolished specific binding (Fig 2B, lane Hm). To assess the specific NF- κ B components in the binding complex, antibody-mediated super-shift assays were performed using antibodies against the five known NF- κ B subunits (p50, p52, p65, cRel and Rel B). As shown in Fig. 2C, both anti-p65 and anti-p50 antibodies retarded the binding complex, suggesting that the detected active NF- κ B complex in hypoxia-treated rat cardiac fibroblasts consists of p65 and p50 subunits.

Active NF- κ B is required for cardiac fibroblast survival under hypoxia

Next, we tested whether inhibition of NF- κ B renders cardiac fibroblasts susceptible to hypoxia. An I- κ B-super-repressor (I- κ B-sr) plasmid was constructed that encoded a mutated I- κ B protein that bound specifically and irreversibly to NF- κ B subunits (p65/p50), preventing its nuclear translocation. Transfected cardiac fibroblasts were subjected to 12 hours of hypoxia or normoxia and cell viability was analyzed by Hoechst 33342 staining. A significant increase in the percentage of dead cells was evident in I- κ B-sr-transfected cells subjected to hypoxia, compared to I- κ B-sr-transfected normoxic cells (Fig. 3A). Cell death due to electroporation *per se* was noted in normoxic I- κ B-sr- and EGFP-transfected control groups (Fig. 3A).

The pharmacological inhibitor, Bay 11-7085, is reported to effectively inhibit NF- κ B activation in different cell types [22, 23]. The inhibitory effect of Bay 11-7085 on NF- κ B was first confirmed in preliminary experiments in the present study (data not shown). Upon exposure of the cells to 4 μ mol/L Bay 11-7085 for 12 hours, a significant increase in the number of PI-positive cells was observed under hypoxic but not normoxic conditions ($p < 0.01$, Figs. 3B & C). Notably, cell death in the control groups was meagre when exposed to Bay 11-7085, in contrast to the control groups subjected to electroporation. Consistent with these early changes, exposure of cardiac fibroblasts to 4 μ mol/L Bay 11-7085 for an extended duration of 24 hours was found to cause extensive morphologically evident cell damage and loss under hypoxic but not normoxic conditions (Fig. 3D), further suggesting that NF- κ B inhibition renders cardiac fibroblasts susceptible to hypoxia.

Preliminary experiments showed that caspase-3 is not induced upon 12 hours of exposure to hypoxia (Fig. 4). Interestingly, cell death in NF- κ B-inhibited hypoxic cells (Figs. 3B & C) was not associated with activation of caspase-3 (Fig. 4), which indicated caspase-independent cell death. Extended exposure to hypoxia (24 hours) did not induce caspase-3 (data not shown).

Regulation of Bcl-2 and cIAP-2 by NF- κ B in hypoxic cardiac fibroblasts

Mayorga et al (2004) examined the expression of several pro- and anti-apoptotic factors such as Bid, Bim, Bax, Bak, Bcl-xL, Smac, xIAP, Apaf-1 and Bcl-2 and attributed an overriding role for Bcl-2 in the resistance of neonatal rat cardiac fibroblasts to different pro-apoptotic stimuli [4]. The study did not, however, examine cIAP-2 status. We therefore assessed the expression of Bcl-2 and cIAP-2 in hypoxic cardiac fibroblasts, and their possible regulation by NF- κ B. Western blot analysis demonstrated comparable constitutive levels of Bcl-2 in cardiac fibroblasts exposed to 12 hours of hypoxic or normoxic conditions (Fig. 5). On the contrary, marked induction of cIAP-2 protein was observed when the cells were subjected to 12 hours of hypoxia (Fig. 6). Similarly, at 6 and 24 hours of hypoxia treatment, only constitutive levels of Bcl-2 were observed but cIAP-2 was significantly higher (data not given).

Normoxic, hypoxic and Bay 11-7085-treated (NF- κ B-inhibited) hypoxic cells had comparable levels of Bcl-2 (Fig. 5), showing that Bcl-2 expression in cardiac fibroblasts is not under NF- κ B control. In contrast, hypoxia-induced cIAP-2 expression was attenuated by Bay 11-7085 (Fig. 6), suggesting the involvement of NF- κ B in regulating cIAP-2 expression in cardiac fibroblasts under hypoxia. Lung fibroblasts hardly expressed Bcl-2 or cIAP-2 protein when subjected to normoxic or hypoxic conditions for 6–24 hours (data not shown), consistent with their susceptibility to hypoxia.

Table 1 sets the various parameters against the treatment durations and summarizes the findings of the study.

Discussion

In the heart, an increase in fibroblast numbers, consequent upon failure of cell turnover mechanisms *post repair*, and associated increments in matrix components can be a major component of myocardial remodeling in several pathological states such as myocardial hypertrophy and hypertensive heart disease [3]. Identification of factors that regulate the size of the cardiac fibroblast population is therefore important for understanding the mechanisms involved in left ventricular remodeling.

In looking for the molecular basis of cardiac fibroblast resistance to hypoxia, we focused on NF- κ B because, apart from its involvement in immune response, embryonic development, cell cycle, apoptosis, inflammation and oncogenesis, [15,24,25] it is a well-characterized stress-activated transcription factor with a major role in cell survival [26]. The first indication of an anti-apoptotic ability of NF- κ B came from analysis of Rel A^{-/-} knockout mice, which died at the embryonic stage due to massive hepatocyte apoptosis [27]. NF- κ B has been reported to prevent TNF- α -induced cell death in mouse embryonic fibroblasts, macrophages and cardiac myocytes [18]. Within the heart, different cell types respond differently to NF- κ B. It has been reported that NF- κ B-dependent activation of survival proteins like Bcl-2 and IAPs protects adult cardiac myocytes against ischemia-induced apoptosis in murine models of acute myocardial infarction [28]. NF- κ B has also been found to mediate cell survival through suppression of pro-apoptotic proteins such as BNIP3 in ventricular myocytes [29]. In human and rat vascular smooth muscle cells, increased NF- κ B expression controls the induction of apoptosis [30]. In contrast, NF- κ B is reported to mediate endothelial cell apoptosis in response to oxidative stress [17, 31]. The involvement of NF- κ B in cardiac fibroblast survival under stress has not been established. The present study shows that NF- κ B may be an important mediator of cell survival in adult rat cardiac fibroblasts exposed to hypoxic stress.

In this study, significant DNA-binding activity of NF- κ B was observed following exposure of the cells to hypoxic conditions for 6 hours (Fig. 2A). The effect of NF- κ B abrogation on cardiac fibroblast survival under hypoxia was examined by a gene-based method wherein a mutated I κ B protein binds and inhibits nuclear translocation of NF- κ B. I κ B-sr-EGFP was constructed from plasmid pLL143 by deleting an intervening poly-A tail that was masking its EGFP expression while retaining its super repressor capability. NF- κ B inhibition by the plasmid-based approach, and by using a pharmacological inhibitor, was found to significantly compromise the viability of cardiac fibroblasts under hypoxic stress (Fig. 3A-D). The findings underscored the dependence of cardiac fibroblasts upon active NF- κ B for survival under hypoxic conditions.

Although the involvement of NF- κ B in the regulation of anti-apoptotic Bcl-2 and cIAP-2 has been reported earlier [32], a link between NF- κ B and these proteins has not been looked into in relation to cardiac fibroblast survival under stress. The present study examined the expression of Bcl-2 and cIAP-2 in normoxic, hypoxic and NF- κ B-inhibited hypoxic cardiac fibroblasts. The pattern of Bcl-2 expression in cardiac fibroblasts subjected to hypoxic and normoxic conditions observed in this study is consistent with the earlier report that constitutive expression of Bcl-2 may be responsible for the resistance of neonatal rat cardiac fibroblasts to diverse insults [4]. It is, however, pertinent to note that Bcl-2 is down-regulated after birth in most tissues [33]. This study extends the earlier observation on neonatal cells and presents evidence that even adult cardiac fibroblasts express Bcl-2 constitutively and that hypoxia does not alter its levels of expression. On the other hand, the present study demonstrated hypoxic induction of cIAP-2 in these cells, consistent with earlier reports that hypoxia selectively up-regulates cIAP-2 expression in other cell types such as 3T3 fibroblasts, primary cultures of HUVEC, human renal carcinoma cells and

human kidney proximal tubular epithelial cells where it confers apoptotic resistance [34, 35].

The Bcl-2 family of anti-apoptotic proteins protects against the mitochondria-dependent death pathway. Bcl-2 sequesters pro-apoptotic Bid from activating Bax/Bak and blocks them from forming pores in the outer mitochondrial membrane and prevents release of pro-apoptotic Smac/DIABLO/AIF/cytochrome C into the cytoplasm [36]. IAPs, on the other hand, directly inhibit active caspases (caspases-3, -7 and -9) to promote cell survival [37] and hence suppress both mitochondria-dependent and -independent apoptosis by inhibiting executioner caspases [38]. The findings presented here support the postulation that Bcl-2 may be involved in the “house-keeping” control of the apoptotic threshold in cardiac fibroblasts whereas cIAP-2 may be a dynamic regulator of apoptosis, induced under hypoxic stress. The pro-survival role of cIAP-2 under hypoxia may be particularly important in light of our recent report that hypoxia causes a dramatic increase in TNF- α production in cardiac fibroblasts that does not compromise fibroblast viability but exerts paracrine effects to promote cardiomyocyte apoptosis [39]. TNF- α is a potent inducer of apoptosis by the extrinsic pathway, which is inhibited by cIAP-2 but not by Bcl-2 [40]. It appears that hypoxic induction of cIAP-2 may stall the mitochondrial death pathway in cardiac fibroblasts at the caspase level and, additionally, it may also block the extrinsic death pathway downstream of TNF- α . Thus, the role of cIAP-2 in cardiac fibroblast survival under hypoxic conditions seems crucial in view of the hypoxic induction of TNF- α . Since TNF- α is known to activate NF- κ B that in turn can help cells resist death by turning on protective proteins like the IAPs [41], it is tempting to speculate that TNF- α may undermine its own pro-apoptotic potential in cardiac fibroblasts by activating NF- κ B and NF- κ B-dependent survival pathways.

Interestingly, Bcl-2 levels were comparable in normoxic, hypoxic and NF- κ B-inhibited hypoxic cells (Fig. 5) but cIAP-2 was strikingly reduced in NF- κ B-inhibited hypoxic cells (Fig. 6). The observation suggests that NF- κ B may regulate cIAP-2 but not Bcl-2 in cardiac fibroblasts under hypoxic stress, which is consistent with earlier reports that cIAP-2 is a downstream target of NF- κ B in other cell types [42]. Although these data do not provide direct evidence that the pro-survival role of NF- κ B is mediated by cIAP-2, hypoxic induction of cIAP-2 in cardiac but not pulmonary fibroblasts, the well-known anti-apoptotic action of cIAP-2 and, importantly, the striking positive correlation between cIAP-2 expression and the activation status of NF- κ B in these cells are consistent with such a postulation. Admittedly, the effect of cIAP2 knockdown, which would be expected to clarify its pro-survival role more definitively, was not examined in the present study. However, it needs to be pointed out that survival strategies in cardiac fibroblasts may be dynamic and may involve multiple factors including, but possibly not limited to, Bcl-2 and cIAP-2. It is unlikely that any single factor or mechanism can explain the totality of cardiac fibroblast resistance to cell death. Thus, cIAP-2 may only be one of the downstream effectors of NF- κ B contributing to its protective role. In this regard, it is important to note that although Bcl-2 was identified as a key element in the death resistance of neonatal rat cardiac fibroblasts, Bcl-2 knockdown by siRNA was found to cause only a small increase in the number of apoptotic cells upon exposure to staurosporine, which was strikingly lower than the fraction of dermal fibroblasts (staurosporine-susceptible “control”) that undergo apoptosis in response to the same stimulus [4].

It is to be noted that hypoxia did not induce caspase-3 in these cells, consistent with the observations of Mayorga et al who reported lack of caspase-3 activation in neonatal rat cardiac fibroblasts exposed to staurosporine and other pro-apoptotic factors, and consequent failure of apoptosis [4]. Stringent regulation of caspase-3 seems an important mechanism by which cardiac fibroblasts resist apoptosis under conditions that compromise the viability of

co-resident cells. Remarkably, cell death in NF- κ B-inhibited hypoxic cells was also not associated with caspase-3 activation. This indicates caspase-independent apoptosis or, alternatively, necrosis resulting from inhibition of a stress response involving NF- κ B whose role in hypoxic cardiac fibroblasts may not be limited to providing apoptosis resistance. Together, the findings suggest that NF- κ B may play an anti-apoptotic role in hypoxic cardiac fibroblasts via its regulatory effects on cIAP-2, but its inhibition in hypoxic cells promotes cell death by a caspase-independent mechanism.

Conclusions

This study generates important leads on the less-explored subject of cardiac fibroblast resistance to stress conditions in the myocardium. We provide evidence for the first time that NF- κ B is a key factor in the survival of cardiac fibroblasts under hypoxic conditions. Further, positive regulation of cIAP-2 may contribute to its pro-survival role in hypoxic cardiac fibroblasts. Identification of other downstream targets of NF- κ B relevant to cell survival under conditions of ambient stress and delineation of NF- κ B-dependent mechanisms that contribute to the relative resistance of cardiac fibroblasts to death signals may uncover mechanisms that enable these cells to resist cell death under stress and play a central role in cardiac remodelling associated with many pathological states of the heart.

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Fig 1A: **Fig.1B:** **Fig.1C:**

Fig.1D:

Fig. 1. Effect of hypoxia on cardiac vs. pulmonary fibroblasts

A: Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were subjected to normoxic (N) or hypoxic (H) treatment for 48 hours. Hypoxia did not produce morphological changes in these cells. Representative phase contrast micrographs from one of six experiments are shown.

B: Sub-confluent cultures of pulmonary fibroblasts in M199 containing 10% FBS were subjected to normoxic (N) or hypoxic (H) treatment for 48 hours. Exposure to hypoxia produced extensive damage. Representative phase contrast micrographs from one of three experiments are shown.

C: Sub-confluent cultures of cardiac fibroblasts in M199 containing 10% FBS were exposed to normoxic (N) or hypoxic (H) conditions for 20 hours and then stained with Hoechst 33342 and PI as described under Methods. Very few cells took up PI, showing that hypoxia does not compromise cardiac fibroblast viability. Representative fluorescent micrographs from one of four experiments are shown.

D: Sub-confluent cultures of pulmonary fibroblasts in M199 containing 10% FBS were subjected to normoxic (N) or hypoxic (H) treatment for 20 hours and then stained with Hoechst 33342 and PI as described under Methods. A marked increase in the number of PI-positive cells showed that hypoxia compromises pulmonary fibroblast viability. Representative fluorescent micrographs from one of two experiments are shown.

Fig.2A

Fig.2B:

Fig.2C

Fig. 2. Hypoxia activates NF-κB in cardiac fibroblasts

Confluent cardiac fibroblast cultures in M199 containing 0.1% FBS were subjected to hypoxic and/or normoxic treatments for 6 hours and nuclear extracts were prepared using the NucBuster Protein extraction kit. Primers for NF-κB were labeled with $\gamma^{32}\text{P}$ -ATP using T4 kinase. After the labeling reaction, the reaction mixtures were cleared with G-50 minicolumns. The nuclear extracts were incubated with the radio labeled probes at room temperature for 90 minutes, and electrophoresed on a 6 % non-denaturing DNA retardation gel at 150V for 50 minutes. The gels were then processed for autoradiography.

A: DNA-binding activity of NF-κB was observed in hypoxic (lane H) but not normoxic (lane N) cardiac fibroblasts. OCT-1 served as loading control. A representative profile from one of five experiments is shown.

B: Competition assay: Nuclear extracts from hypoxic cells were subjected to electrophoresis with $\gamma^{32}\text{P}$ -labelled NF-κB oligos with an excess of un-labelled wild and mutant oligos, in separate reaction mixtures, and autoradiography was performed. NF-κB bands were not seen

in the presence of the wild-type NF- κ B oligo (lane Hw) due to competition. A representative profile from one of three experiments is shown.

C: Super-shift assay was performed using antibodies against the five known NF- κ B subunits (p50, p52, p65, cRel and Rel B). Arrows correspond to the shifted p65 and p50 NF- κ B bands. A representative profile from one of three experiments is shown.

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D

Fig. 3. Effect of NF- κ B inhibition on cell viability

A: The I- κ B -super repressor, I- κ B-sr-EGFP, was constructed from plasmid pLL143 as described under Methods. I- κ B-sr-transfected cardiac fibroblasts in M199 with 10% FBS were subjected to 12 hours of hypoxia or normoxia and then stained with Hoechst 33342. A significant increase in the percentage of dead cells was observed in I- κ B-sr-transfected cells subjected to hypoxia. Values are expressed as Mean $\pm$ SD. H-I κ B vs N-I κ B, ** p < 0.001. “n” indicates the number of dishes and a minimum of 250 cells were counted per dish.

B: Sub-confluent cardiac fibroblast cultures in M199 with 10% FBS were exposed to 12 hours of normoxia (N) or hypoxia (H) in the presence or absence of 4 μ mol/L Bay-11-7085. Cell death was assessed by Hoechst 33342-PI staining as described under Methods. A marked increase in the number of PI-positive cells was observed in Bay-11-7085-treated-cells under hypoxic (HBay) but not normoxic (NBay) conditions. Representative fluorescent micrographs from one of three experiments are shown.

C: Sub-confluent cardiac fibroblast cultures in M199 with 10% FBS were exposed to 12 hours of normoxia (N) or hypoxia (H) in the presence or absence of 4 μ mol/L Bay-11-7085. Cell death was assessed by Hoechst 33342-PI staining as described under Methods. A significant increase in the percentage of PI-positive cells was evident in cultures exposed to hypoxia for 12 hours in presence of 4 μ mol/L Bay-11-7085 (HBay). Values are expressed as Mean $\pm$ SD. HBay vs NBay, ** p < 0.01. “n” indicates the total number of dishes and a minimum of 250 cells were counted per dish.

D: Confluent cardiac fibroblast cultures in M199 with 10% FBS were exposed to normoxia (N) or hypoxia (H) for 24 hours in the presence or absence of 4 μ mol/L Bay-11-7085. Extensive cell damage was noticed in cells exposed to hypoxia in the presence of 4 μ mol/L

Bay-11-7085 (HBay). Representative phase contrast micrographs from one of two experiments are shown.

Fig. 4. Effect of hypoxia and Bay 11-7085 on caspase-3 activity in cardiac fibroblasts

Caspase-3 activity was assessed as described under Methods in confluent cardiac fibroblast cultures in M199 with 10% FBS exposed to 12 hours of hypoxia (H) or normoxia (N), with or without 4 μ mol/L Bay 11-7085 (Bay). Activity is expressed in arbitrary units. Caspase-3 activity was not affected by the treatments. Values are expressed as Mean $\pm$ SD of three separate experiments.

Fig. 5. Effect of hypoxia and Bay 11-7085 on Bcl-2 expression in cardiac fibroblasts

Confluent cultures of cardiac fibroblasts in M199 with 10% FBS were exposed to normoxia (N) or hypoxia (H) for 12 hours in the presence or absence of 4 μ mol/L Bay-11. Western blot analysis, performed as described under Methods, using monoclonal anti-Bcl-2 antibody showed comparable levels of Bcl-2 in normoxic (N), hypoxic (H) and NF- κ B-inhibited (4 μ mol/L Bay-11-7085) hypoxic (HBay) cells. A representative profile from one of four experiments is shown.

Fig. 6. Effect of hypoxia and Bay 11-7085 on cIAP-2 expression in cardiac fibroblasts

Confluent cultures of cardiac fibroblasts in M199 with 10% FBS were exposed to normoxia (N) or hypoxia (H) for 12 hours in the presence or absence of 4 μ mol/L Bay-11. Western blot analysis was performed as described under Methods, using polyclonal anti-cIAP-2 antibody. Significant induction of cIAP-2 expression was observed in hypoxic cells (H), which was attenuated in NF- κ B-inhibited (4 μ mol/L Bay-11-7085) hypoxic cells (HBay). A representative profile from one of three experiments is shown.

Table 1

Relative resistance of cardiac fibroblasts to hypoxia			
Hypoxia treatment duration		Parameter	
48hours		Cell morphology	Pulmonary fibroblasts – Extensive damage
20hours		PI uptake	Cardiac fibroblasts – No damage
			Pulmonary fibroblasts - +ve
			Cardiac fibroblasts - -ve

Effects of hypoxia on cardiac fibroblasts			
Treatment	6hours	12hours	24hours
Hypoxia	NF-κB↑ cIAP-2↑ Bcl-2 (constitutive)	cIAP-2↑ Bcl-2 (constitutive) Caspase (-ve) PI uptake (-ve)	cIAP-2↑ Bcl-2 (constitutive) Caspase (-ve) Phase contrast: No damage
Hypoxia + NF-κB inhibitor	NF-κB↓ Bcl-2 (constitutive)	cIAP2↓ Bcl-2 (constitutive) Caspase (-ve) PI uptake (+ve)	Phase contrast: Extensive damage