A direct requirement of nuclear factor-κB for suppression of apoptosis in ventricular myocytes

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Mustapha, Shareef, Alla Kirshner, Danielle De Moissac, and Lorrie A. Kirshenbaum. A direct requirement of nuclear factor-κB for suppression of apoptosis in ventricular myocytes. Am J Physiol Heart Circ Physiol 279: H939–H945, 2000.—Nuclear factor-κB (NF-κB) is a ubiquitously expressed cellular factor regulated by the cytoplasmic factor inhibitor protein κBα (IκBα). Activation of NF-κB by cytokines, including tumor necrosis factor-α (TNF-α), requires the phosphorylation and degradation of IκBα. An anti-apoptotic role for NF-κB has recently been suggested. In the present study, we ascertained whether death-promoting signals and apoptosis mediated by TNF-α are suppressed by NF-κB in postnatal ventricular myocytes. Stimulation of myocytes with TNF-α resulted in a 12.1-fold increase (P < 0.01) in NF-κB-dependent gene transcription and DNA binding compared with controls. This was accompanied by a corresponding increase in the NF-κB target protein A20 as determined by Western blot analysis. Vital staining revealed that TNF-α was not cytotoxic to myocytes and did not promote apoptosis. Adenovirus-mediated delivery of a nonphosphorylatable form of IκBα to inactivate NF-κB prevented TNF-α-stimulated NF-κB-dependent gene transcription and nuclear NF-κB DNA binding. Importantly, myocytes stimulated with TNF-α and defective for NF-κB activation resulted in a 2.2-fold increase (P < 0.001) in apoptosis. To our knowledge, the data provide the first indication that a functional NF-κB signaling pathway is crucial for suppressing death-promoting signals mediated by TNF-α in ventricular myocytes.

adenovirus; inflammation; cytokines; heart failure

PROGRAMMED CELL DEATH is a highly conserved evolutionary event crucial for normal development and homeostasis. Deregulated cell death has been associated with disease entities such as cancer (36, 42), human immunodeficiency virus infection (29), and more recently cardiovascular disease (22, 38). Notably, apoptosis has been detected in cardiac tissue after ischemia followed by reperfusion (17), oxidative stress injury (15), postinfarction (20), and in patients with end-stage heart failure (37, 41). Because ventricular myocytes retain a limited regenerative potential after birth, the loss of potentially viable cells through an apoptotic process may profoundly influence cardiac structure/function. Although the molecular mechanisms that govern apoptosis in the heart are poorly defined, there is increasing awareness that certain cellular factors can either promote or suppress the cell death process.

Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor that is regulated by the inhibitor protein κBα (IκBα). IκBα binds to and sequesters NF-κB in the cytoplasm, preventing NF-κB from translocating to the nucleus. Signal-induced activation of NF-κB involves the phosphorylation of IκBα at serine residues 32 and 36. This leads to the ubiquitination and degradation of IκBα by the proteasome, allowing NF-κB to translocate to the nucleus and affect gene transcription (14). Recently, an anti-apoptotic function for NF-κB has been described (5, 50, 52). This is supported by studies in which cells defective for NF-κB signaling were found to be more sensitive to proapoptotic signals than normal wild-type cells (49, 50). Furthermore, transgenic mice incapable of NF-κB activation die at embryonic day 14.5 from excessive apoptosis and severe liver degeneration (46). Together, these observations support a critical role for NF-κB in abrogating death-promoting signals and apoptosis.

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine with diverse biological functions that include cell proliferation, inflammation, and apoptosis. Although TNF-α is known to strongly activate NF-κB, there is emerging evidence suggesting that TNF-α predominately triggers apoptosis in cells that are either deficient or defective for NF-κB activation (5, 49, 50). The relative spatial and temporal expression of TNF-α in the heart, particularly during heart hypertrophy and end-stage heart failure (cf. Refs. 21, 33), raises the possibility that TNF-α directly modulates NF-κB activity and the apoptotic process. However, whether NF-κB suppresses death-promoting signals mediated by TNF-α in ventricular myocytes is unknown and has not been formally tested. Therefore, in the present study, we examined the significance of the NF-κB signaling pathway in ventricular myocytes by determin-
ing whether a block to NF-κB activation would unmask the cytotoxic actions of TNF-α and render ventricular myocytes susceptible to apoptosis. In this report, we provide the first direct evidence to support a role for NF-κB as an anti-apoptotic factor in ventricular myocytes. Furthermore, our data show that a functional NF-κB signaling pathway is crucial for preventing death-promoting signals and apoptosis in ventricular myocytes mediated by TNF-α.

METHODS

Cell culture and transfection. Neonatal ventricular myocytes were isolated from 2-day-old Sprague-Dawley rat hearts and were submitted to primary culture as described previously (9). After an overnight incubation in DMEM-Ham’s nutrient mixture F-12 (1:1), 17 mM HEPES, 3 mM NaHCO₃, 2 mM L-glutamine, 50 μg/ml gentamicin, and 10% fetal bovine serum (FBS), cells were transferred to serum-free medium as previously described (9, 24). Myocytes were infected with recombinant adenoviruses and transfected after removal of viral stocks with NF-κB luciferase reporter plasmid in DMEM containing DEAE-dextran as previously described (9). Myocytes were stimulated with 10 ng/ml of human recombinant TNF-α (R&D Systems) or 10 μM C₂₁ ceramide (Sigma Chemical, St. Louis, MO) in serum-free media for 24–72 h. This concentration of ceramide was previously shown to trigger apoptosis of ventricular myocytes (26). Luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency and was expressed as relative light units. Data were obtained from at least n = 3 independent myocyte cultures with replicates of three for each condition. Results were compared by Student’s t-test, using a significance level of P ≤ 0.05.

Recombinant adenoviruses. Adenoviruses were propagated, harvested, purified, and titered from 293 cells as previously reported (23). The cDNA epitope-FLAG-tagged derivative of the IκBα mutant containing serine-to-alanine substitutions at amino acid positions 32 and 36 was generously provided by D. Ballard (6) and was subcloned into the Hind III/Xba I sites of an adenovirus shuttle plasmid. Recombinant adenovirus was generated by homologous recombination in 293 cells as previously reported (10, 23). Viral infection was controlled for by using the adenovirus designated AdCMV, which contains the cytomegalovirus (CMV) enhancer-promotor without a cDNA insert. Myocyte cultures were infected with 20 plaque-forming units per cell of recombinant adenovirus for 4 h. This titer of virus achieves gene delivery to ≥95% of neonatal ventricular cells under these conditions (23).

Western blot analysis. For immunodetection of IκBα and A20 proteins, cardiac myocytes were harvested in buffer containing 0.5% SDS, 150 mM NaCl, and 50 mM Tris·HCl, pH 7.4 (RIPA buffer). Cell lysates (100 μg) were resolved on a 10% SDS-polyacrylamide gel at 140 V for 4 h and were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics). For detection of IκBα-FLAG-tagged proteins, the PVDF filter was incubated with a rabbit antibody directed toward IκBα clone C2 (1 μg/ml; Santa Cruz Biotechnology) or a polyclonal antibody directed toward human A20 protein (25). Bound proteins were visualized by chemiluminescence reaction with horse-radish peroxidase-conjugated antibodies against mouse or rabbit IgG using enhanced chemiluminescence reagents (Amersham).

Electromobility gel shift assay. Nuclear extracts of cardiac myocytes were prepared as previously described by de Moisac et al. (9) with modifications. Briefly, 3 × 10⁶ cells were pelleted and resuspended in 200 μl of 10 mM HEPES, pH 7.9, 60 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, protease inhibitors, and 0.3% Nonidet P-40. Cells were allowed to swell on ice for 15 min and then were centrifuged at 1,000 g at 4°C. The supernatant was extracted, and the remaining cell pellet was resuspended in 50 μl of 200 mM HEPES, pH 7.9, 0.4 M NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride at 4°C for 15 min. The nuclear extract was centrifuged for 5 min at 10,000 g and was stored at −80°C. Analysis of DNA-binding activities by electromobility shift analysis was carried out as previously described using a 32P-radiolabeled duplex oligonucleotide probe containing NF-κB consensus binding sites 5′-AGTGGAGGGGACTTTCGCAGGC-3′. DNA binding reactions (20 μl) were carried out on ice and contained 5 μg nuclear extract, 2 μg double-stranded probe poly(dI·dC) (Pharmacia), 10 μg BSA in 20 mM HEPES, pH 7.9, 5% glycerol, 1 mM EDTA, and 5 mM dithiothreitol. Nuclear-protein complexes were resolved on a 5% polyacrylamide gel in 1× Tris-borate-EDTA (pH 8.0) and were detected by autoradiography.

Viability analysis. Total cell number was determined before and after stimulation with TNF-α to ensure that cells did not aberrantly detach from plates and that equivalent cell numbers were available for viability analysis. Myocytes stimulated with TNF-α were assessed for viability by staining cells with the vital dyes calcein-acetoxymethyl ester (AM) (2 μM) and ethidium homodimer-1 (2 μM) and counted by flow cytometry (22, 24). Cells were washed and mounted on glass slides and visualized using an Olympus AX70 Research microscope equipped with an excitation and emission filter set to simultaneously detect the number of live (green) and dead (red) cells, respectively. The relative number of green vs. red cells was determined from at least ≥200 cells/condition.

Detection of apoptosis. Nuclear morphology and nucleosomal DNA fragmentation of cardiac nuclei were determined by staining myocytes with Hoechst 33258 dye for nuclear DNA as previously described (22, 24). Replicate cultures using ≥200 cells for each condition were utilized. Genomic DNA was isolated from ventricular myocytes for nucleosomal DNA fragmentation by gel electrophoresis as previously described (22, 24).

Statistical analysis. Data were obtained from at least n = 3 independent cell cultures with replicates of three for each condition. Results were compared by Student’s t-test, using a significance level of P ≤ 0.05.

RESULTS

To establish whether ventricular myocytes are functionally coupled to biological signals that lead to the downstream activation of NF-κB by TNF-α, ventricular myocytes were transfected with a luciferase reporter gene containing putative binding sites for NF-κB and were stimulated with TNF-α (9). A 12-fold increase (P < 0.01) in NF-κB-dependent gene transcription was observed in the presence of TNF-α compared with vehicle-treated control cells (Fig. 1). Moreover, stimulation of myocytes with TNF-α resulted in a threefold induction of the endogenous A20 protein, a protein known to be regulated by NF-κB (25, 43; Fig. 2). Furthermore, gel shift experiments indicated that NF-κB...
binding activity was increased in myocytes stimulated with TNF-\(\alpha\) compared with vehicle-treated control cells (Fig. 3, lane 2 vs. lane 3). Moreover, competition binding assays with 100-fold excess probe (lane 7) as well as supershift experiments with antibodies directed toward the p65 subunit of NF-\(\kappa\)B (lane 6) confirmed that the higher migrating complex contained the p65 subunit of NF-\(\kappa\)B. Together these findings confirm that ventricular myocytes are functionally coupled to biological signals that link TNF-\(\alpha\) to NF-\(\kappa\)B DNA binding and NF-\(\kappa\)B-dependent gene transcription.

To formally test whether TNF-\(\alpha\) is cytotoxic and provokes apoptosis of ventricular myocytes, ventricular myocytes were stimulated with TNF-\(\alpha\) and stained with the vital dyes calcein-AM and ethidium homodimer-1 to identify the live and dead cells, respectively. As shown in Fig. 4A, myocytes stimulated with TNF-\(\alpha\) for up to 72 h were indistinguishable from vehicle-treated control cells with respect to cell viability (\(P = 0.31\)), indicating that TNF-\(\alpha\) alone was not cytotoxic to myocytes and did not provoke cell death. Importantly, no significant difference in cell number was observed after stimulating cells with TNF-\(\alpha\) at any of the time points tested, verifying that cells were not preferentially lost by TNF-\(\alpha\) stimulation (Fig. 4B).
contrast, ventricular myocytes stimulated with the cell-permeable sphingolipid C2 ceramide (10 μM), a by-product of TNF-α stimulation in ventricular myocytes (39) known to provoke apoptosis at the concentration utilized (26), resulted in widespread cell death compared with vehicle-treated cells or those stimulated with TNF-α (Fig. 4A). This substantiates that ceramide, but not TNF-α, was toxic to myocytes.

However, it has recently been shown that the cytotoxic responses to TNF-α can be enhanced by agents such as actinomycin D or cycloheximide, which inhibit transcription and translation, respectively (30). The fact that these agents unmask the cytotoxic actions of TNF-α suggests that the de novo activation of downstream genes that are cytoprotective are important for preventing TNF-α-mediated cell death.

In this regard, the transcription factor NF-κB has been suggested to be important in preventing death-promoting signals and apoptosis mediated by TNF-α. Because NF-κB activity is governed by IκBα, which binds to and sequesters NF-κB in the cytoplasm, we determined whether NF-κB is necessary for suppressing apoptosis in ventricular myocytes by testing whether a block to NF-κB activation with a mutant form of IκBα would render ventricular myocytes susceptible to TNF-α-induced cell death. For these experiments, we generated a replication defective adenovirus encoding an IκBα molecule containing serine-to-alanine point substitutions at amino acid positions 32 and 36, respectively. This renders IκBα defective for phosphorylation and degradation, thereby preventing signal-induced nuclear activation of NF-κB (3, 4). As shown by electromobility gel shift analysis, TNF-α-mediated NF-κB nuclear DNA binding was inhibited to basal levels in cells expressing the IκBα mutant (IκBMT) and those defective for NF-κB activation.

Together these findings verify that the IκBα mutant was functionally active in ventricular myocytes in suppressing signal-induced activation of NF-κB by TNF-α. Importantly, myocytes infected with the control adenovirus were not different from uninfected control cells with respect to viability (Fig. 4A), confirming that adenoviral infection was not toxic to myocytes (24). In contrast, cells expressing the IκBα mutant and stimulated with TNF-α displayed a significant 2.2-fold increase in the incidence of cell death compared with
vehicle-treated control cells or those stimulated with TNF-α (Fig. 4C; P < 0.001). Moreover, genomic DNA isolated from myocytes expressing the mutant IκBα and stimulated with TNF-α displayed evidence of apoptosis, as demonstrated by an increase in nucleosomal DNA laddering (Fig. 5). Similarly, myocytes defective for NF-κB activation and stimulated with TNF-α displayed characteristic features of apoptosis by Hoechst 3325 dye compared with control cells (de Moissac and Kirshenbaum, unpublished observation). Together, these findings support our contention that activation of the NF-κB signaling pathway is crucial for suppressing death-promoting signals and apoptosis in ventricular myocytes that would otherwise be provoked by TNF-α.

DISCUSSION

To our knowledge, the data provide the first direct evidence for the operation of the NF-κB signaling pathway for the suppression of apoptosis induced by TNF-α in ventricular myocytes. Furthermore, our data indicate that TNF-α alone does not provoke apoptosis in ventricular myocytes that are functionally coupled to the downstream activation of NF-κB. A role for TNF-α as a modulator of cardiac function has been proposed and substantiated by the relative spatial and temporal expression of this cytokine in the myocardium but notably by its upregulation during mechanical load (47) in surviving myocytes after infarction (35) and end-stage heart failure (31, 48). The unexpected and counterintuitive lack of apoptosis in ventricular myocytes with TNF-α, despite evidence elsewhere (11, 26), suggests that TNF-α likely activates dual signaling cascades in a cell- and context-specific manner, with one pathway leading to apoptosis while the other pathway, mediated through NF-κB, dominates to suppress prodeath signals and apoptosis (5, 34, 51).

The physiological/pathophysiological role of TNF-α in the heart is unknown. However, the fact that TNF-α does not provoke apoptosis in the presence of a functional NF-κB signaling pathway seen here suggests that TNF-α may have an alternative role as a stress response factor (19, 32). In this regard, recent in vitro and in vivo studies have shown that TNF-α can modulate contractile function (21) and gene expression (12) characteristic of the dilated failing heart (27). Moreover, because elevated TNF-α levels have been detected in cardiac pathologies such as allograft rejection (45), postinfarction, end-stage heart failure (47), and viral myocarditis (28), it is tempting to speculate that NF-κB contributes to the inflammatory response of these conditions by suppressing or blunting the apoptotic response of cells to TNF-α (2, 13). This notion is supported by recent studies in which endothelial cells, key mediators of the inflammatory response, were found to be resistant to TNF-α, whereas endothelial cells defective for NF-κB activation readily underwent apoptosis provoked by TNF-α (1, 40). These observations are consistent with the findings of the present study which demonstrate that interference with signal-induced activation of NF-κB unmasks the cytotoxic effects of TNF-α, resulting in apoptosis of ventricular myocytes.

The mode by which NF-κB suppresses apoptosis is unknown but may be related to the activation of downstream genes that regulate the apoptotic process. This is supported by the fact that several anti-apoptotic factors, including cellular inhibitors of apoptosis (c-IAP), c-IAP1, c-IAP2, (7), A20 (8), and IEX-1L (53), are known transcriptional targets of NF-κB. These factors can reportedly block the activation of caspase 8, the proximal caspase in the TNF-α/CD95/Fas signaling pathway that propagates apoptotic signals through activation of the death-inducing signaling complex (44).

Whether these factors are present and functionally active in ventricular myocytes is unknown. However, the fact that cell-permeable C2 ceramide but not TNF-α was cytotoxic to myocytes affirms the notion that activation of signaling molecules downstream of TNF-α receptor are crucial for suppressing prodeath signals and apoptosis (16). Although our data substantiate a cytoprotective role for NF-κB in ventricular myocytes, it must be stated that protection from apoptosis may not be a universal feature of NF-κB, since under certain instances such as the case with Sindbis virus, NF-κB may trigger rather than prevent apoptosis (18). Furthermore, the role played by NF-κB in cardiac disease conditions is unknown and awaits further investigation. Thus whether NF-κB operates as a pro- or anti-apoptotic factor may rely on the context of cell.
type and the ensuing stimulus. Nevertheless, under the conditions tested, our data provide the first direct evidence to support a role for the suppression of TNF-α-mediated apoptosis in ventricular myocytes by NF-κB and highlights the importance of coordinated regulation of NF-κB by TNF-α to modulate the apoptotic response during disease states. Our current investigations are directed toward elucidating the impact of the NF-κB signaling pathway and the downstream effector proteins that regulate apoptosis in cardiac disease conditions.

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REFERENCES


