Modulation of Cytokine-Induced Cardiac Myocyte Apoptosis by Nitric Oxide, Bak, and Bcl-x

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Abstract—Cytokine-induced NO production depresses myocardial contractility and has been shown to be cytotoxic to cardiac myocytes. However, the mechanisms of cytokine-induced cardiac myocyte cell death are unclear. To analyze these mechanisms in detail, we treated neonatal cardiac myocytes in serum-free culture with a combination of the macrophage-derived cytokines interleukin-1β, tumor necrosis factor-α, and interferon-γ. These cytokines caused a time-dependent induction of cardiac myocyte apoptosis, but not necrosis, beginning 72 hours after treatment, as determined by nuclear morphology, DNA internucleosomal cleavage, and cleavage of poly(ADP-ribose) polymerase, reflecting caspase activation. Apoptosis was preceded by a >50-fold induction of inducible NO synthase mRNA and the release of large amounts (5 to 8 nmol/μg protein) of NO metabolites (NOx) into the medium. Cell death was completely blocked by an NO synthase inhibitor and attenuated by antioxidants (N-acetylcysteine and DTT) and the caspase inhibitor ZVAD-fmk. Cytokines also mediated an NO-dependent, sustained increase in myocyte levels of the Bcl-2 homologs Bak and Bcl-x(L). The NO donor S-nitrosoglutathione also induced apoptosis and cell levels of Bak, but not of Bcl-x(L). All effects of cytokines, including poly(ADP-ribose) polymerase cleavage, could be attributed to interleukin-1β, interferon-γ and tumor necrosis factor-α had no independent effects on apoptosis or on NOx production. We conclude that cytokine toxicity to neonatal cardiac myocytes results from the induction of NO and subsequent activation of apoptosis, at least in part through the generation of oxygen free radicals. The rate and extent of this apoptosis is modulated by alterations in the cellular balance of Bak and Bcl-x(L), which respond differentially to cytokine-induced and exogenous NO and by the availability of oxidant species. (Circ Res. 1999;84:21-33.)

Key Words: poly(ADP-ribose) polymerase ■ protein kinase G ■ nitric oxide ■ Bcl-x(L) ■ oxidative stress

Nitric oxide, a short-lived free radical–generating gas, is an important signal-transduction molecule in many cell types, regulating such diverse functions as vasomotor tone, neurotransmission, mediation of immune responses, and inflammatory cell adhesion to the vessel wall.1–3 In the cardiac myocyte, the major physiological role of NO appears to involve depression of contractility and electrophysiological stabilization through elevation of intracellular cGMP.2,4–6 A rise in myocardial NO levels may account for the contractile depression observed during sepsis7,8 and in isolated cardiac myocytes following exposure to macrophage-conditioned medium9,10; endogenous NO production has been shown to mediate the negative chronotropic effects of carbachol in cardiac myocytes.11

NO is generated by 2 different enzymes in myocardium, which are macrophage-type, or inducible NO synthase (iNOS), and endothelial-type NO synthase (eNOS).12,13 iNOS differs from eNOS in being constitutively active and regulated primarily through transcriptional means, while eNOS is present but inactive until exposed to elevated cytosolic calcium. Most cardiac myocyte NO is thought to be produced by iNOS. iNOS is strongly induced in cardiac myocytes exposed to macrophage-derived cytokines, including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-1β,10,12,14–18 and during allograft rejection, in which cytokines are present in high levels.19 This strong induction may be attributed to the presence of more than 22 immune effector–responsive elements in the iNOS promoter, including binding sites for nuclear factor κB and IFN regulatory factor-1.20

The high levels of NO produced by iNOS are cytotoxic and are an important part of the host defense against microbial invasion in many species. Cytokine-induced NO is lethal to cardiac myocytes in vitro21 and to several other cell types.22–27 In vivo models of cardiac allograft rejection show a correlation between induction of iNOS and increased apoptosis,21,28 while forced expression of human eNOS in rat myocardium induces features of apoptosis in transfected cardiac myocytes and neighboring cells.29 Elevation of iNOS in chronic congestive heart failure may contribute to myocar-
dial dysfunction and disease progression. However, the effects of NO are highly tissue specific and may promote survival in specific cell types, including endothelial cells.

Recently, we showed that atrial natriuretic peptide (ANP), via a cGMP-dependent pathway, induces cardiac myocyte apoptosis, accompanied by increased expression of the apoptosis modulator Mcl-1. We reasoned that NO should also activate programmed cell death, since cGMP mediates many of the effects of NO in other cell types. The experiments presented here were designed to determine the contribution of NO to cytokine-induced cardiac myocyte death and to determine whether NO effector systems alter expression of apoptosis-modulating genes in the heart.

Materials and Methods

Expression vectors encoding Bcl-2, Bax, Bak, and Bcl-x(L/S), and a monoclonal antibody to Bak, were the kind gift of Dr Michael Kiefer (LXR Biotechnology, Richmond, Calif). The cDNA probe for murine macrophase iNOS was generously provided by Dr Charles Lowenstein (The Johns Hopkins University, Baltimore, Md). Antibodies to Bcl-x(L/S), Bax, and Bcl-2 were obtained from Oncogene Science (Uniondale, NY). The human GAPDH cDNA was purchased from Clontech Laboratories (Palo Alto, Calif).

Hoechst 33342 and propidium iodide dyes were purchased from Molecular Probes. Human TNF-α and rat IFN-γ were obtained from Genzyme (Cambridge, Mass). Recombinant human IL-1β and the protein kinase G-specific inhibitor KT5823 were obtained from Calbiochem (La Jolla, Calif). All other reagents were purchased from Sigma except where indicated and were of the highest grade available.

Cell Culture

All procedures involving animals were performed in accordance with institutional guidelines for the care and use of animals. Methods for primary culture of neonatal rat cardiac myocytes have been previously described. In brief, enriched cultures of myocyte and nonmyocyte cells were obtained from 1- to 2-day-old neonatal rats by stepwise trypsin dissociation and plated at a density of 4 × 10^6/60-mm dish, or in 6-well plates at a comparable density (2 × 10^5/m²). Cells were examined for morphological evidence of apoptosis or necrosis after staining with Hoechst 33342 and propidium iodide (M20 MU). Myocytes were confirmed by costaining with Hoechst 33342 and a monoclonal antibody against sarcomeric myosin heavy chain, Bak cytochemistry, while propidium iodide-stained cells with normal nuclear morphology were scored as necrotic. To quantify apoptosis, an average of 500 nuclei from random fields were analyzed, and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted. Samples were numbered to conceal the identity of different treatment groups during scoring, and at least 3 samples were scored in each group.

Immunofluorescent Staining

In some experiments, apoptotic nuclei were localized within cardiac myocytes by costaining with Hoechst 33342 and a monoclonal antibody against sarcomeric myosin heavy chain. Cardiac myocytes were cultured on uncoated Nunc 2-well coverslip dishes and treated for 96 hours with IL-1β. Control cells received an equal volume of diluent. Cells were subsequently fixed in ice-cold methanol, rinsed, and stained with anti-myosin heavy chain (MF-20, obtained from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) and Hoechst 33342, followed by an FITC-tagged anti-mouse IgG secondary antibody. Cells were imaged and photographed on a Zeiss IM inverted phase fluorescence microscope using a mounted Contax 35-mm camera and ASA 400 Kodak color transparency film.

Analysis of DNA Fragmentation

Nuclear DNA was isolated from and examined for nucleosomal fragmentation at predetermined time points after treatment with cytokines and other reagents. Cells were lysed overnight in a buffer containing 100 mmol/L NaCl, 10 mmol/L Tris-Cl, pH 8, 25 mmol/L EDTA, 0.5% SDS, and 0.15 mg/mL proteinase K. Lysates were extracted with phenol/chloroform and precipitated with ammonium acetate in 100% ethanol. The resulting pellet was resuspended and treated with DNase-free RNase before reextraction and precipitation as above. Nuclear DNA was then solubilized in Tris-EDTA buffer, and DNA content was quantified by spectrophotometry at 260 nm. Samples were then subjected to electrophoresis in 2% agarose gels and imaged by ethidium bromide staining and digital photography.

In some experiments, before electrophoresis, DNA (1 μg) from each sample was 3’-end labeled using 25 U terminal transferase (Boehringer Mannheim) in a reaction buffer consisting of 0.2 mmol/L potassium cacodylate, 25 mmol/L Tris-HCl, 250 μg/mL BSA, 5 mmol/L CoCl₂, and 50 μCi [α-32P]ddATP at 37°C for 1 hour. Reactions were terminated by addition of EDTA, and DNA was precipitated twice more in ethanol. Samples were then subjected to electrophoresis, dried on a slab gel dryer, and exposed to x-ray film for 2 hours at −70°C. In all cases, the extent of DNA fragmentation was quantified by densitometry of subchromosomal DNA fragments on digitized images using Adobe Photoshop 4.0 for Macintosh.

Northern Blot Analyses

RNA transcript levels were measured by Northern analysis using modifications of a standard protocol. Total cellular RNA was extracted using the Trizol RNA isolation kit (Gibco-BRL) or by guanidinium isothiocyanate and cesium banding. Electrophoresis, blotting, and hybridizations were all as described previously. cDNA probes were labeled with 5’-[32P]ATP by random priming (Prime-It, Stratagene) to 10^6 cpm/μg DNA. Hybridization signals of specific mRNAs were normalized to those of 28S rRNA to correct for differences in loading and/or RNA transfer.

Quantitative Analysis of Apoptotic Nuclei

Cells were examined for morphological evidence of apoptosis or necrosis after staining with the fluorescent DNA-binding dyes H33342 and propidium iodide, as previously described. Treated and control cell monolayers were rinsed with PBS and stained with 5 μg/mL H33342 and 5 μg/mL propidium iodide for 15 minutes and then harvested by trypsinization. Suspended cells were pelleted at 2000g, resuspended in MEM, and visualized at ×400 on a Zeiss IM fluorescence microscope. Cells were scored as apoptotic if they exhibited unequivocal nuclear chromatin condensation and/or fragmentation, while propidium iodide-stained cells with normal nuclear morphology were scored as necrotic. To quantify apoptosis, an average of 500 nuclei from random fields were analyzed, and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted. Samples were numbered to conceal the identity of different treatment groups during scoring, and at least 3 samples were scored in each group.

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Figure 1. Correlation between morphological appearance of apoptosis and NO synthesis in cardiac myocytes. A, Apoptosis induction by cytokines. A fixed combination of the macrophage-derived cytokines TNF-α, IL-1β, and IFN-γ (TNF-α, 25 ng/mL; IL-1β, 5 ng/mL; and IFN-γ, 100 U/mL) was added to cardiac myocytes cultured on glass coverslip dishes alone or in combination, with and without L-NMMA, a competitive inhibitor of iNOS. Control myocytes received the diluent or L-NMMA alone. At the indicated times between 0 and 120 hours after treatment, myocytes were harvested by trypsinization and stained with Hoechst 33342 and propidium iodide, and the percentage of apoptotic nuclei (apoptotic nuclei/total nuclei) was determined for a minimum of 20 microscopic fields (7500 cells).

B, Cytokine stimulation of NOx production. NOx production (nmol/mg protein) was measured spectrophotometrically using the Griess reagent as described in Materials and Methods. The time course of NOx production was determined for a minimum of 20 microscopic fields (7500 cells).

C, Induction of iNOS mRNA by cytokines. Cardiac myocytes were treated with the same cytokine combination as in Figure 1A, with or without L-NMMA. Control cells received vehicle (water) only. Total RNA content was determined using a standard colorimetric assay (BCA, Pierce Chemical). One hundred micrograms of each lysate supernatant was fractionated on 12% SDS-polyacrylamide gels and electroblotted to nitrocellulose (Hybond, Amersham). Gels were stained with Ponceau Red for control for equal transfer of proteins. Membranes were blocked for 1 hour at room temperature with 5% nonfat milk in Tris-buffered saline (25 mmol/L Tris and 150 mmol/L NaCl) containing 0.05% Tween 20 and incubated with antibodies specific to Bcl-x(L) (0.5 μg/mL, Santa Cruz Biotechnology) or Bak (1.0 μg/mL) for 2 hours in the same buffer. After washing, the blots were incubated for 1 hour with a 1:7500 dilution of horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (Bcl-x(L)) or HRP-conjugated donkey anti-goat IgG (Bak) and visualized using an enhanced chemiluminescence detection system (Amersham).

Statistical Analysis

Results were expressed as mean±SEM. Differences between means were evaluated by 2-tailed Student’s t test. ANOVA was carried out using InStat 2.0 statistical software for Macintosh.

Results

Morphological Study of Cytokine-Induced Apoptosis

The time course of cell death in cytokine-treated neonatal rat cardiac myocytes was evaluated by staining the cells as described in Materials and Methods. Both apoptotic and necrotic cells were scored. Under control conditions in DSF medium, myocyte cultures contained a low percentage of apoptotic cells (Figure 1A, •). Treatment of the cells with a mean±SEM of at least 5 determinations in each of 5 experiments. Cytokines were TNF-α, IL-1β, and IFN-γ. C, Induction of iNOS mRNA by cytokines. Cardiac myocytes were treated with the same cytokine combination as in Figure 1A, with or without L-NMMA. Control cells received vehicle (water) only. Total RNA was harvested at the indicated time points as described in Materials and Methods. Shown is a single representative RNA blot probed sequentially with iNOS and GAPDH cDNAs. Cyk indicates cytokines (TNF-α, IL-1β, and IFN-γ); V, vehicle (control).
Figure 2. Cytokine-induced DNA laddering involves NO. A, Apoptosis-associated nucleosomal laddering requires NO synthesis. DNA was extracted from cardiac myocytes between 0 and 120 hours as indicated after addition of vehicle (control), cytokines, or cytokines plus L-NMMA. Samples (1 μg) were end labeled with terminal transferase and subjected to 1% agarose gel electrophoresis as described in Materials and Methods. Nucleosomal cleavage products were visualized by enhanced chemiluminescence. Cyk, indicates cytokines; V, vehicle; and LN, L-NMMA. B, Both cytokines and GSNO induce nucleosomal laddering in cardiac myocytes. Top, DNA was extracted at time points from 0 to 120 hours after treatment with cytokines, as indicated, or 24 hours after treatment with GSNO (1 mmol/L). DNA samples (1 μg) were directly subjected to electrophoresis on 2% agarose gels and imaged by ethidium bromide staining and digital photography. Note the progression from larger (lane 3) to smaller (lane 11) DNA cleavage products between 24 and 120 hours in the presence of cytokines (+). – indicates absence of cytokines. Bottom, Quantification of DNA cleavage. Shown are the mean pixel densities of subchromosomal DNA bands in each lane of the image at top, after background subtraction. C, Inhibition of laddering by methylene blue. Cardiac myocytes were treated with vehicle (C) or cytokines (CYK) with and without methylene blue (MB);
combination of cytokines (5 ng/mL IL-1β, 100 U/mL IFN-γ, and 25 ng/mL TNF-α) produced a time-dependent increase in the percentage of apoptotic nuclei over untreated cells (Figure 1A). The time of onset of cytokine-induced apoptosis varied between 48 and 72 hours (compare Figures 1A, 2A, and 3A). In dual-staining experiments using an anti-desmin antibody together with Hoechst 33342, these apoptotic cells were clearly identified as cardiac myocytes (not shown).

As seen in Figure 1A, the increase in the percentage of apoptotic myocytes was statistically significant at 72 hours (19.1 ± 3.5% versus 4.9 ± 1.5%, n = 3, P < 0.05) and remained nearly 8-fold greater than control at 120 hours (18.5 ± 3.4% versus 2.5 ± 0.8%, n = 3, P < 0.02). Over the same interval, there was no increase in the small (<1%) percentage of necrotic cells (not shown), and there was a gradual decline in the number of control myocytes undergoing spontaneous apoptosis (Figure 1A). The time of onset of cytokine-induced apoptosis varied between 48 and 72 hours.

High levels of NO have been shown to be cytotoxic to many cell types. To determine whether production of NO accounted for the proapoptotic effects of cytokines, we used a competitive inhibitor of NO synthase, N-monomethyl-L-arginine (L-NMMA). An excess concentration of L-NMMA (10 mmol/L) had no effect on basal levels of apoptosis; however, L-NMMA blocked induction of apoptosis by cytokines (Figure 1A).

**Cytokine-Induced Myocyte Apoptosis Correlates With NO Production**

Macrophage-derived cytokines have been shown to exert negative inotropic and toxic effects on cardiac myocytes through the transcriptional activation of iNOS and subsequent production of NO, N-monomethyl-L-arginine (L-NMMA). An excess concentration of L-NMMA (10 mmol/L) had no effect on basal levels of apoptosis; however, L-NMMA blocked induction of apoptosis by cytokines (Figure 1A).

**Cytokines Induce Myocyte DNA Laddering via NO**

DNA cleavage into nucleosome-sized fragments is a hallmark of apoptosis and results from the caspase-induced activation of a specific DNase. To confirm the above results, DNA isolated from cytokine-treated and control cells was end labeled and analyzed on agarose gels as described in Materials and Methods. Control cells exhibited a low level of DNA fragmentation that declined over the course of the experiment. In contrast, DNA from cytokine-treated cells exhibited a clear nucleosomal ladder that was detectable within 48 hours after exposure and was completely eliminated by L-NMMA (Figure 2A). In a second series of experiments, the NO donor S-nitrosoglutathione (GSNO, 1 mmol/L) induced death in 30% to 50% of the cells, which was associated with apoptotic nucleosomal cleavage (Figure 2B and 2D); the total number of affected cells was similar to that for cytokines, but the kinetics appeared to be more rapid, as indicated by the extent of nucleosomal cleavage at 24 hours (Figure 2B, last lane) and by direct identification of apoptotic cells (28.3 ± 3.5%, 1 mmol/L GSNO, versus control percentage of 5.2 ± 0.5% at 4 hours; n = 6, P < 0.0001).

We have previously shown that the cell-permeable cGMP agonist (8-Br-cGMP) directly induces myocyte apoptosis. To further characterize the cellular effects of cytokines, we used methylene blue, an NO scavenger and soluble guanyl cyclase inhibitor, and the cGMP-dependent protein kinase inhibitor KT5728 to attempt to block cytokine-induced DNA laddering, using densitometric quantification of DNA cleavage products as an index. KT5728 did not inhibit, and in some cases potentiated, cytokine-mediated apoptosis but had no independent toxicity (Figure 2C). In contrast, methylene blue, although cytotoxic during prolonged exposure, consistently blunted the apoptotic effects of cytokines (Figure 2C).

Additional evidence for a non-cGMP-dependent mechanism was observed with GSNO. GSNO caused DNA laddering at 4 hours after treatment, in a dose-dependent manner (Figure 2D). The effects of GSNO on apoptosis were maximal at 1 mmol/L, the highest dose tested (Figure 2D, lane 6), and were significantly reduced by cotreatment with 2 different antioxidants, N-acetylcysteine (NAC; Figure 2D, lane 8) and DTT (lane 10). These data are consistent with a direct role for NO in cytokine-mediated apoptosis that may not require activation of cGMP-dependent protein kinase (eg, formation of peroxynitrite).

An important feature of apoptosis is the sequential activation of specific IL-converting enzyme-like proteases, or caspases. Activation of the terminal protease, caspase-3 (CPP32, apopain, MACH-1, or Yama) causes breakdown of key cell substrates and activation of a specific nucleosomal DNase, which produces the characteristic DNA ladder.
IL-1β Induces Cardiac Myocyte Apoptosis via NO

![Graph](Image)

**Figure 3.** IL-1β is required for cytokine-induced apoptosis. A. Induction of apoptosis by IL-1β. Macrophage-derived cytokines TNF-α (25 ng/mL), IL-1β (5 ng/mL), and IFN-γ (100 U/mL) were added separately or in combination to cardiac myocytes cultured on glass coverslip dishes. Control myocytes received the diluent alone. At the indicated times after treatment, myocytes were harvested, stained, and visualized exactly as described in Figure 1A. Values are expressed as percentage apoptotic cells. SEM (n=4). Statistically significant increases in apoptosis occurred beginning at 48 hours for cytokine combinations containing both IL-1β and IFN-γ (with and without TNF-α), **P<0.005** for comparisons between IL-1β alone and IL-1β+IFN-γ. Cyk indicates TNF-α+IL-1β+IFN-γ; control, diluent only. B. Stimulation of NO production by IL-1β alone and IL-1β+IFN-γ. Cyk indicates TNF-α+IL-1β+IFN-γ; control, diluent only. Each data point represents the mean±SEM of at least 5 determinations in each of 5 experiments. Combinations containing both IL-1β and IFN-γ induced NO production more rapidly than IL-1β alone. **P<0.05** for comparisons between IL-1β alone and IL-1β+IFN-γ.

Inhibition of caspase activity by the peptide pseudosubstrate ZVAD-fmk strongly attenuated GSNO-induced apoptosis (Figure 2D, lane 7). These data support the requirement for formation of oxidative NO products such as peroxynitrite in the induction of apoptosis by cytokines and GSNO, as well as the activation of caspases during this process.

**IL-1β Alone Induces Apoptosis**

The cytokines used in this study and elsewhere include 3 proteins with distinct characteristics. IFN-γ has been shown to act independently to induce apoptosis in many cell types; TNF-α and related molecules induce apoptosis by a specific receptor-mediated, Fas-dependent mechanism, and TNF-α has been shown to induce apoptosis in isolated adult cardiac myocytes. The actions of IL-1β are more complex and include both hypertrophic and antiproliferative effects depending on the cell type. To better define the signal mechanisms involved in cytokine-induced apoptosis, we examined the independent effects of IL-1β, TNF-α, and IFN-γ on the appearance of apoptotic nuclei. IL-1β alone was sufficient to activate apoptosis (Figure 3A). A statistically significant increase in apoptosis was achieved earlier with the combination of IL-1β and IFN-γ (48 hours) than with IL-1β alone (Figure 3A, compare △ and ●; *P<0.05 at 48 hours). This difference was offset by a relatively higher rate of apoptosis in the IL-1β-treated cells at 96 and 120 hours (*P<0.05, IL-1β versus IL-1β+IFN-γ). These results indicate that IFN-γ accelerates the onset of apoptosis induced by IL-1β. However, neither TNF-α nor IFN-γ had independent proapoptotic effects on cardiac myocytes (Figure 3A).

The ability of specific cytokines to induce apoptosis was closely tied to their ability to induce NO synthesis. As shown in Figure 3B, IL-1β stimulated a rapid sustained increase in NOx. As with apoptosis, IFN-γ increased the production of NO by IL-1β at early (48 hours) but not late (96 hours) time points (Figure 3B, △ versus ●; *P<0.05 at 48 hours). However, IFN-γ had no effect on NO production or apoptosis by itself. In contrast, TNF-α had no effect on NO production whether alone or in addition to the other cytokines (Figure 3B, ∆). Thus, the ability of each cytokine to induce NO.
production correlated closely with its ability to induce apoptosis (Figure 3, compare panels A and B). Moreover, the onset of apoptosis after cytokine treatment closely followed the appearance of significant levels of NO metabolites in the culture medium.

**IL-1β Mimics Cytokine-Induced pADPRp Cleavage**

A well-characterized substrate of caspase-3 is pADPRp, which maintains the integrity of chromosomal DNA. To confirm the activation of apoptotic pathways in cytokine-treated myocytes, we examined cell lysates for pADPRp cleavage products (Figure 4). In control cells, bands corresponding to both cleaved and uncleaved pADPRp were identified, with uncleaved protein predominating. This ratio shifted markedly in favor of the 85-kDa cleavage product after treatment with either IL-1β (Figure 4, top) or all 3 cytokines (Figure 4, bottom). These data confirm that cytokines activate cysteiny1 proteases associated with apoptosis and that IL-1β by itself is sufficient for this activation.

**Both Proapoptotic and Antiapoptotic Genes Are Upregulated by Cytokines**

The relative abundance of cell survival-regulating factors encoded by the Bcl-2 gene family can strongly affect, if not determine, the decision to undergo apoptosis in response to an external signal. Both proapoptotic and antiapoptotic Bcl-2 homologs have been identified in myocardium; evidence suggests that several of these proteins and/or their mRNA transcripts are regulated during myocardial development and aging as well as following ischemic stress. However, it is not clear what molecular effectors regulate the abundance of these proteins. We previously found that myocyte McI-1 levels were downregulated in response to ANP treatment, coincident with the onset of apoptosis. We therefore attempted to determine whether cytokines and/or NO also influenced the balance of cell survival factors.

In Northern analyses, Bak and Bcl-x(L) transcripts were present in untreated cells and rose significantly within 24 hours after treatment with both cytokines and IL-1β alone.
(Figure 5). In contrast, mRNA transcripts for the related proteins Bcl-2 and Bax were undetectable in both control and cytokine-treated myocytes (not shown). Furthermore, although both Bcl-2 and Bax proteins were identified on Western blots, their levels were not modulated by cytokines (not shown).

The increase in Bak and Bcl-x(L) transcripts was accompanied by induction of Bak and Bcl-x(L) proteins as
determined by Western analysis (Figure 6A through 6D). A doublet band migrating at 30 kDa was visualized by the Bcl-x antibody; no smaller protein bands were detected by this or by a second Bcl-x(S)-specific antibody (not shown), suggesting absence of the proapoptotic alternatively spliced product. The cytokine-mediated increases in both Bak and Bcl-x(L) were NO dependent, as they were blocked by L-NMMA (Figure 6B, lane 7, and Figure 6D, ○). IL-1β by itself was sufficient to induce Bcl-x (Figure 6C, lanes 6 to 10, and Figure 6D, ▲) and Bak (Figure 6D, bottom panel), with kinetics similar to those of cytokines.

Surprisingly, however, the NO donor GSNO did not induce expression of Bcl-x(L) either at 24 hours (Figure 6C, lanes 12 and 13) or at 4 hours (Figure 7), at times when DNA laddering was easily detected (see Figure 2B and 2D). In contrast, GSNO (1 mmol/L) induced Bak by 2.0-fold over control (P<0.0001) by 4 hours after addition to the cultured cells (Figure 6D). This increase was quantitatively similar to the induction of Bak by IL-1β at 48 hours and resulted in a significant shift in the ratio of Bak to Bcl-x(L) in favor of apoptosis.

**Discussion**

Apoptosis has been strongly implicated in the toxicity of NO for cardiac myocytes. This study provides definitive evidence that cytokine-induced NO production transduces a proapoptotic signal in cardiac myocytes, as defined by morphological criteria, DNA laddering, and pADPRp cleavage. Our data further show that cytokines increase cardiac myocyte expression and abundance of Bak and Bcl-x(L), 2 proteins with opposing roles in the modulation of apoptosis. Interestingly, we were unable to induce apoptosis in cardiac fibroblasts obtained from the same cultures (not shown), suggesting that the induction of apoptosis was cell type specific, occurring predominantly or exclusively in cardiac myocytes. Significantly, the induction of apoptosis by ANP was also selective for cardiac myocytes in these mixed cultures. Since cardiac myocytes and fibroblasts both possess ANP receptors and soluble guanylate cyclase, it is likely that factors distal to these receptors render myocytes sensitive to ANP- and NO-induced apoptosis.

Several lines of evidence suggest that NO is, in fact, the effector of both cytokine-mediated apoptosis and Bak/Bcl-x induction. (1) The ability of individual cytokines to induce NO production in cardiac myocytes exactly paralleled their ability to activate programmed cell death. (2) The competitive iNOS antagonist, L-NMMA, prevented both NO production and apoptosis as determined by morphological criteria and DNA laddering and also blocked the expression of Bak and Bcl-x. (3) Methylene blue, an NO scavenger and inhibitor of soluble guanylate cyclase, attenuated cytokine and IL-1β-induced DNA laddering. (4) An NO donor, GSNO, rapidly induced myocyte-specific apoptosis. (5) Cytokine- and IL-1β-dependent induction of both Bak and Bcl-x were entirely blocked by L-NMMA, indicating that, like apoptosis, the regulation of these apoptosis-modulating factors was also dependent on NO.

NO exerts its effects on tissues through several mechanisms. It activates guanylate cyclase, resulting in an increase in intracellular cGMP; it modulates the L-type calcium channel current; and it reacts with oxygen free radicals to generate peroxynitrite, a potent oxidant. We have previously demonstrated that other cGMP agonists, including the atrial and brain natriuretic peptides zaprinast and 8-Br-cGMP, are proapoptotic in cardiac myocytes. Oxidative stress is also a potent inducer of apoptosis, and redox state influences both cardiac myocyte signal transduction and gene transcription. The present studies argue in favor of an oxidative, rather than a cGMP-dependent, mechanism for cytokine/NO-mediated apoptosis in these neonatal rat cells. Most importantly, KT5823, a cGMP-dependent protein kinase-1 inhibitor, failed to prevent cytokine/NO-mediated apoptosis, while having no independent toxic effects. Although methylene blue inhibits soluble guanylate cyclase, this inhibition is most likely due to reactive destruction of NO through free radical generation. Thus, the inhibition of cytokine-mediated apoptosis by methylene blue can be directly attributed to quenching of NO but not necessarily to inhibition of guanylate cyclase. Since the degree to which NO forms peroxynitrite is dependent on the availability of oxygen.

**Figure 7.** GSNO induces Bak but not Bcl-x protein. Cardiac myocytes were treated with GSNO at the indicated concentrations for 4 hours, and lysates were subjected to Western analysis with specific Bak and Bcl-x antibodies as described in Materials and Methods. Top, Representative Western blots. Bcl-x indicates lysate of cardiac myocytes transfected with Bcl-x expression vector; Bcl-2, lysate of cardiac myocytes transfected with Bcl-2 expression vector. Bottom, Summary of 3 experiments. Densitometric data have been normalized to control levels. Error bars=SEM. *P<0.0001 vs control.
free radicals, the redox state of individual myocytes may be an important variable in determining the apoptotic response to NO. This possibility is supported by the fact that the antioxidants NAC and DTT were each able to inhibit GSNO-induced apoptosis (see Figure 2D). Further studies will be required to determine the relative importance of these mechanisms in the cardiac effects of NO.

The process of apoptosis appears to require both an initial extrinsic signal and a complex intrinsic gene-regulated apoptotic program. Modulation of the abundance of apoptosis regulators of the Bcl-2 family plays a critical role in the determination of cell fate in response to apoptotic stress. These neonatal myocytes expressed predominantly Bcl-x(L) and Bak; low levels of Bcl-2 and Bax were not altered by cytokine treatment (not shown). Unlike Bcl-2, Bak is proapoptotic; the mechanisms of Bak-associated cell death are incompletely understood, but they appear to involve both caspase-dependent and -independent pathways. Induction of Bak by NO would thus be predicted to potentiate cytokine-induced apoptosis.

In contrast, transcriptional induction of Bcl-x(L) is likely to be antiapoptotic. Bcl-x(L) protects against apoptosis in many cell types in response to a wide range of apoptosis effectors and following Fas-induced protease activation. In none of the experiments did we detect transcripts or protein corresponding to the proapoptotic splice variant Bcl-x(S). Bcl-x(L) acts upstream of the initiation of apoptosis and acts to delay its onset or reduce its probability, in part through altering mitochondrial membrane permeability. One transcriptional regulator of Bcl-x(L) is the tumor suppressor protein p53, and induction of Bcl-x in this setting could thus involve NO-dependent upregulation of p53. p53 upregulates Bcl-x(L) in the setting of DNA damage, possibly serving to delay the onset of apoptosis long enough to permit repair of salvageable cells. By analogy, NO-dependent, cytokine-mediated Bcl-x(L) induction might allow cardiac myocytes to survive transient or physiological rises in NO.

It is interesting in this regard that GSNO evoked apoptosis, DNA laddering, and Bak expression without induction of Bcl-x(L). The reason for the distinct effects of GSNO and cytokines on Bcl-x expression is not clear, but they may be due to differences in the kinetics or quantity of NO production by the 2 agents and differences in the regulation of the 2 genes. NO release from GSNO is rapid and affects all cells simultaneously, while NO production in response to cytokines requires a finite delay for transcription and protein synthesis. It is also possible that cytokine-activated signals in addition to NO generation are required for the induction of Bcl-x(L), but not Bak. The result is that the NO donor produces a relative increase in proapoptotic effector proteins compared with cytokines, and this may account in part for the difference in the speed and extent of apoptosis in response to the 2 agents. This difference also supports a role for Bcl-x(L) in promoting myocyte survival after cytokine exposure as proposed above.

The presence of reactive oxygen is a critical determinant of how NO behaves in biological systems. In a recent study, late myocardial preconditioning was mimicked by 2 different NO donors; protection was abolished by antioxidant treatment, suggesting that formation of peroxynitrite or OH was required for the beneficial effects of NO. In another study a third NO donor was shown to protect against stretch-induced superoxide production, contractile abnormalities, and programmed cell death. Both beneficial and potentially deleterious effects of NO and its oxidative products have thus been demonstrated in different systems. Differences in the rate and timing of NO release, as well as in the species and other experimental conditions, may account for the observed differences in the effects of NO. However, reactive oxygen concentration is clearly important. It is known that the presence of reactive oxygen determines the specific products of NO donor molecule breakdown (NO versus NO3 versus NO2), as well as the reaction products and ultimately the protein targets of NO in the cell. It is likely that the seemingly inconsistent behavior of NO in these different studies is related to the strong dependence of NO signaling events on cell redox state.

In this study, induction of apoptosis was limited to those cytokine combinations that generated NO. However, TNF-α has been reported to activate cardiac myocyte apoptosis through cell surface receptors of the TNF receptor family. TNF receptor ligands are able to activate the cysteine protease cascade directly, using specific adapter proteins (reviewed in References 78 and 79). However, TNF-α was neither necessary nor sufficient to induce apoptosis (or necrosis) in neonatal cardiac myocytes, in agreement with the earlier study. This finding demonstrates the primacy of NO in mediating neonatal rat cardiac myocyte apoptosis, as TNF-α at this dose was also unable to stimulate iNOS transcription or NO production (Figure 2 and data not shown). It is possible that neonatal myocytes lack 1 or more components required for TNF-α signal transduction. Consistent with this hypothesis, transcripts for the type I TNF receptor have been reported to be undetectable in cultured neonatal cardiomyocytes, in contrast to adult myocytes. This would explain the lack of NO production by TNF-α in these cells.

Whether TNF-α can induce cardiac myocyte apoptosis through the induction of NO or other mechanisms in vivo remains to be determined. Interestingly, most primary cell cultures are resistant to TNF-mediated cytotoxicity, except under special circumstances such as expression of viral oncogenes, oncogenic transformation, or inhibition of protein synthesis. Indeed, TNF-α was recently shown to induce hypertrophy rather than apoptosis in adult cardiac myocytes in a manner that depended on intact cell-substrate interaction. Although a line of transgenic mice overexpressing TNF-α in myocardium develops contractile abnormalities, this occurs on the background of a chronic inflammatory state and in the absence of significant apoptosis. The role of apoptosis and of other TNF-α-mediated effector systems in the development and progression of congestive heart failure in vivo remain to be established.
IL-1β, in contrast, appears to be the key effector of cytokine-mediated apoptosis in these cells, consistent with its ability to induce NOx. IFN-γ, while having no independent proapoptotic or antiapoptotic effect, appeared to alter the kinetics of IL-1β–induced apoptosis without changing the total number of apoptotic cells over the course of the experiment. The proapoptotic properties of IL-1β are distinct from its previously reported trophic effect on cardiac myocytes, which is mediated by a tyrosine kinase–dependent pathway.48,49 This observation is interesting in light of the hypothesis that sustained hypertrophic stress leads to apoptosis88 and the possibly conflicting view that growth factors may present a means to treat heart failure.84–87 Data presented here and elsewhere suggest that, in fact, the downstream signal pathways regulating cardiac myocyte hypertrophy and apoptosis are dissociable. For example, the antiapoptotic effects of β-adrenergic stimulation are calcium independent, while β-adrenergic trophic effects require calcium entry.36,40 Similarly, the antiapoptotic and trophic effects of cardiotrophin-1 are mediated by distinct intracellular entry.36,40 Similarly, the antiapoptotic and trophic effects of cardiotrophin-1 are mediated by distinct intracellular effectors.88 The precise relationship between cardiac myocyte growth and death signals, and their effectors, remains to be elucidated.

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