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

Douglas J. Ing, Jie Zang, Victor J. Dzau, Keith A. Webster, Nanette H. Bishopric

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 expression 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.11 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 myocardial dysfunction,30,31 which is associated with depressed glucose metabolism and 32,33 decreased expression of eNOS. Our data show that NO is not required for cytokine-induced apoptosis in cardiac myocytes. Instead, NO acts as a proximate mediator of the effector mechanisms that promote cell death. This effect of NO is consistent with various observations showing NO to play a role in cell death, including induction of apoptosis by NO donors in T lymphocytes,19,34,35 neuronal cultures,36 and 37,38 vascular smooth muscle cells.39–41 Moreover, in this study, we show that NO is required for cytokine-mediated expression of the prosurvival proteins Bak and Bcl-x(L). This suggests that NO induction of apoptosis is linked to the expression of proapoptotic proteins, such as Bak and Bcl-x(L). This is consistent with a recent study showing Bcl-x(L) expression to be increased in cells treated with NO-donating compounds.42 These findings are consistent with the hypothesis that NO has a dynamic role in mediating cell survival or death. In this regard, NO not only acts as an activator of apoptosis, as shown in our study, but also as an inhibitor of apoptosis, as demonstrated by other investigators.43,44

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

Received August 10, 1998; accepted October 20, 1998.

From the Departments of Molecular and Cellular Pharmacology and Medicine (J.Z., K.A.W., N.H.B.), University of Miami School of Medicine, Miami, Fla; Falk Cardiovascular Research Center (D.J.I.), Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, Calif; and Department of Medicine (V.J.D.), Brigham and Women’s Hospital, Boston, Mass.

Correspondence to Nanette H. Bishopric, MD, FACC, Associate Professor of Pharmacology and Medicine, Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine (R-189), PO Box 016189, Miami, FL 33101. E-mail nhb@chroma.med.miami.edu

© 1999 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org
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

Materials

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 macrophage 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 iode 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 non-myocyte cells were obtained from 1- to 2-day-old neonatal rats by stepwise trypsin dissociation and plated at a density of 4×10⁷-60 mm dish, or in 6-well plates at a comparable density (2×10⁶ cells/cm²), in MEM supplemented with 5% FCS, penicillin, and streptomycin (MEM 5% FCS). On the following day, cells were examined for morphological evidence of apoptosis or necrosis after staining with the fluorescent DNA-binding dyes Hoechst 33342 and propidium iodide, as previously described. Treated cells were examined for morphological evidence of apoptosis or necrosis by staining with the fluorescent DNA-binding dyes Hoechst 33342 and propidium iodide, as previously described. Mixtures of equal volumes of medium and Griess reagent were mixed and incubated at room temperature for 30 minutes. The red diazotization product was quantified by spectrophotometry at 570 nm using known concentrations of sodium nitrite as a standard.

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-mysin myosin heavy chain (MF-20, obtained from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) and Hoechst 33342, followed by an FITC-tagged 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 8.5, 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 mol/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.

NO Analysis

The Griess reagent (2.9 mol/L sulfanilic acid and 0.2 mol/L N-(1-naphthyl) ethylenediamine-HCl in 5% phosphoric acid) was used to measure the accumulation of NO metabolites (predominantly NO₂⁻) as a proxy for NO synthesis. Equal volumes of medium and Griess reagent were mixed and incubated at room temperature for 30 minutes. The red diazotization product was quantified by spectrophotometry at 570 nm using known concentrations of sodium nitrite as a standard.

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 ≥10⁶ 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.
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 (×500 cells). Each experiment was performed 5 times. Values are expressed as percentage apoptotic cells ± SEM (n = 5). *P < 0.05 for cytokine-treated vs control cells. B, Cytokine stimulation of NO production. NO reaction products (NOx, nmol/mg protein) were measured spectrophotometrically using the Griess reagent as described in Materials and Methods. Both apoptotic and necrotic nuclei was scored. Under control conditions in DSF medium, myocyte cultures contained a low percentage of necrotic cells (<1% of the total) and no apoptotic cells. Cytokines were TNF-α, IL-1β, and IFN-γ. B, Cytokine stimulation of NO production. NO reaction products (NOx, nmol/mg protein) were measured spectrophotometrically using the Griess reagent as described in Materials and Methods. Cytokines were TNF-α, IL-1β, and IFN-γ. Each experiment was performed 5 times. Values are expressed as NOx production (nmol/mg protein) ± SEM (n = 5). Statistical analysis was carried out using ANOVA and Tukey’s test. ANOVA was carried out using InStat 2.0 statistical software for Macintosh.

**Statistical Analysis**

Results are 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);
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.\(^{45}\) 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.\(^{36}\) To further characterize the cellular effects of cytokines, we used methylene blue, an NO scavenger and soluble guanyl cyclase inhibitor,\(^{44}\) 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,\(^{45}\) which produces the characteristic DNA ladder.

10 μmol/L or KT5728 (KT; 1 μmol/L) as indicated. Subchromosomal DNA was quantified for each condition by densitometry as described for panel B, above, and is expressed in arbitrary densitometry units±SEM (n=3) obtained from 3 separate experiments. n.s. = P>0.1. D, Dose-dependent effects of GSNO on cardiac myocyte apoptosis. The NO donor GSNO was added to cardiac myocyte cultures for 4 hours at the indicated concentrations, with and without the caspase inhibitor ZVAD-fmk (50 μmol/L) or antioxidants (NAC, 10 mmol/L, or DTT, 1 mmol/L). Cells were then harvested for analysis of DNA fragmentation as described in Materials and Methods. ZVAD indicates ZVAD-fmk. Data are expressed as mean±SEM. *P<0.05; **P<0.001.
IL-1β Induces Cardiac Myocyte Apoptosis via NO

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 at 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

![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 vehicle 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=5). 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: ▲ and ○, respectively), whereas apoptosis increased beginning at 72 hours for IL-1β alone. *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. NOx species were quantified as described in Figure 1B. Medium was sampled from cardiac myocytes between 0 and 120 hours after stimulation with the individual cytokines or combinations used in Figure 3A. Each data point is 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.

![Figure 4. Both cytokines and IL-1β induce pADPRp cleavage. The proteolytic cleavage of pADPRp was assayed at various times after treatment of cardiac myocytes with IL-1β (top) or the cytokine combination (Cyk) described in Figure 1 (bottom). Myocyte lysates were subjected to Western blot analysis using an anti-pADPRp antibody provided by Dr Guy Poirier. Caspase-activated proteolysis of pADPRp is inferred from the disappearance of the uncleaved 116-kDa polypeptide and a relative or absolute increase in the larger of 2 cleavage products at 85 kDa. Human HL-60 progranulocytic cells, untreated (U) or induced with cycloheximide (l), were used as controls. Slight differences in band sizes between HL-60 and cardiac cells probably reflect a species difference between rat and human. The Western blot shown is representative of 3 experiments with similar results.]
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 cysteinyl 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 Mcl-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.
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 shown in Figure 6. Induction of apoptosis-modulating proteins by NO agonists. A, Induction of Bcl-x(L) and Bak by cytokines. Protein lysates were extracted from cardiac myocytes between 0 and 120 hours after treatment with the combination of cytokines IL-1β, TNF-α, and IFN-γ as described in Materials and Methods. Specific bands are indicated by arrows. No band corresponding to the alternative splice product Bcl-x(S) was seen. B, Cytokine-mediated Bcl-x induction requires NO. Shown is a Western blot of cardiac myocyte lysates at 0 and 48 hours after treatment with vehicle, L-NMMA, cytokines, or a combination of these. Cyk indicates cytokines (TNF-α, IL-1β, and IFN-γ); LN, L-NMMA; and V, vehicle. C, IL-1β, but not an NO donor, induces Bcl-x. Cardiac myocytes were treated in parallel with cytokines, IL-1β alone, or the NO donor GSNO (GS). D, Comparison of Bak and Bcl-x induction by cytokines and IL-1β. Blots were analyzed by densitometry as described in Materials and Methods. Data are expressed in arbitrary densitometry units; each data point represents results obtained in at least 3 experiments. *P<0.05 for cytokine-treated vs control cells; **P<0.05 for IL-1β-treated vs control cells.
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). Two 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.
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-3-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. The reaction products and ultimately the protein targets of NO determine the specific products of NO donor molecule breakdown (NO 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. This observation is interesting in light of the hypothesis that sustained hypertrophic stress leads to apoptosis and the possibly conflicting view that growth factors may present a means for treating heart failure.

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.

Similarly, the antiapoptotic and trophic effects of cardiotrophin-1 are mediated by distinct intracellular effectors. The precise relationship between cardiac myocyte growth and death signals, and their effectors, remains to be elucidated.

Acknowledgments

This work was supported by NIH grants HL49891 (to N.H.B.) and HL44578 (to K.A.W.), by grant 4RT-0134 from the California Tobacco-Related Diseases Research Program (to K.A.W. and N.H.B.), and by an American Heart Association Established Investigator Grant (to N.H.B.). Dr Ing is supported by a Research Fellowship from the Medical Research Council of Canada. We are grateful to Daryl Discher and Barbara Sato for expert technical assistance and to Dr Matt Pollman for help with the DNA ladderning protocol. We also thank Drs Robert Furchgott and Larry Boise for helpful suggestions and discussions.

References

32  

IL-1β Induces Cardiac Myocyte Apoptosis via NO


71. Klefström J, Arigui E, Littlewood T, Jäättela M, Saksela E, Evan GI, Alitalo K. Induction of TNF-sensitive cellular phenotype by c-Myc...


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

Douglas J. Ing, Jie Zang, Victor J. Dzau, Keith A. Webster and Nanette H. Bishopric

Circ Res. 1999;84:21-33
doi: 10.1161/01.RES.84.1.21

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/84/1/21

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/