Cytokine-Mediated Apoptosis in Cardiac Myocytes
The Role of Inducible Nitric Oxide Synthase Induction and Peroxynitrite Generation

Margaret A. Arstall, Douglas B. Sawyer, Ryuji Fukazawa, Ralph A. Kelly

Abstract—Increased production of nitric oxide (NO) after induction of the cytokine-inducible isoform of nitric oxide synthase (iNOS or NOS2) in cardiac myocytes and other parenchymal cells within the heart may in addition to contributing to myocyte contractile dysfunction also contribute to the induction of programmed cell death (apoptosis). To investigate the mechanism(s) by which increased NO production leads to apoptosis, we examined the role of NO in primary cultures of neonatal rat ventricular myocytes (NRVMs) after induction by the cytokines interleukin-1β (IL-1β) and interferon γ (IFNγ) or exposure to the exogenous NO donor S-nitroso-N-acetylcycteine (SNAC) or peroxynitrite (ONOO⁻). Both SNAC (1 mmol/L) and ONOO⁻ (100 μmol/L), but not their respective controls (ie, N-acetylcycteine and pH-inactivated ONOO⁻), induced apoptosis in confluent, serum-starved NRVMs at 48 hours. Similarly, incubation of NRVMs with IL-1β and IFNγ for 48 hours resulted in an increase in iNOS expression, nitrite production, and programmed cell death. Both the cytokine-induced nitrite accumulation and myocyte apoptosis could be completely prevented by the nonselective NOS inhibitor L-nitroarginine (3 mmol/L) or the specific iNOS inhibitor 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT, 100 μmol/L). NO-mediated myocyte apoptosis was not attenuated by the inhibition of soluble guanylyl cyclase with ODQ, nor could apoptosis be induced by the incubation of NRVMs with 1 mmol/L 8-bromo-cGMP, a cell-permeant cGMP analogue. However, NO-mediated apoptosis was significantly attenuated by the superoxide dismutase mimetic and ONOO⁻ scavenger Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP, 100 μmol/L). NO/ONOO⁻-mediated apoptosis was associated with increased expression of Bax with no change in Bcl-2 mRNA abundance. Furthermore, apoptotic cell death was also confirmed in adult rat ventricular myocytes (ARVMs) when grown in heteroculture with IL-1β- and IFNγ-treated rat cardiac microvascular endothelial cells. Therefore, cytokine-induced apoptosis in NRVMs and ARVMs is mediated by iNOS induction, ONOO⁻, and associated with an increase in Bax levels. (Circ Res. 1999;85:829-840.)

Key Words: nitric oxide • peroxynitrite • cytotoxicity • apoptosis • programmed cell death

Increased production of nitric oxide (NO) after induction of inducible nitric oxide synthase (iNOS or NOS2) has been implicated in the pathophysiology of myocardial dysfunction of a number of disease syndromes, including the systemic inflammatory response syndrome, inflammatory myocarditis, cardiac allograft rejection, and some forms of heart failure. Cardiac myocytes, as well as a number of other parenchymal cells within the myocardium, including the endothelium of the coronary microvasculature and the endocardium, and infiltrating inflammatory cells are all able to express iNOS in response to soluble inflammatory mediators, including specific cytokines and bacterial cell-wall components. In the presence of adequate levels of cofactors (heme, tetrahydrobiopterin) and substrates (O₂ and l-arginine), very high levels of NO production can be achieved after iNOS induction, levels that may limit growth or prove lethal to invading pathogens, underscoring the importance of iNOS induction to the innate immune response. These levels of NO production may be detrimental to myocardial function, however, as iNOS induction has been demonstrated to diminish both basal and catecholamine-enhanced chronotropic and inotropic function in isolated myocytes and in the intact heart. The mechanisms by which NO mediates these effects include increased activation of soluble guanylyl cyclase and an increase in cGMP, inhibition of electron transport by the mitochondrial respiratory chain, and nitrosation of functionally important thiol residues on proteins, and the production of oxidants, such as peroxynitrite (ONOO⁻).

iNOS induction within the heart, as in other tissues and organs, may subserve an additional role as part of the innate immune response—programmed cell death or apoptosis of infected cells. NO generated by iNOS has been implicated in the induction of apoptosis in a number of cell types.
including cardiac myocytes. Ing et al. determined that iNOS-induced cardiac myocyte apoptosis appeared to be independent of cGMP and was possibly mediated by the generation of oxygen-derived free radicals. It is not known to what extent NO itself, or ONOO' contributes to iNOS-dependent cell death in cardiac myocytes.

In this study, we confirm the data of Ing et al. that a combination of interleukin-1β (IL-1β) and interferon γ (IFNγ) induces programmed cell death associated with increased Bax relative to Bcl-2 expression in isolated neonatal rat ventricular myocytes (NRVMs). We show that the mechanism is at least partially dependent on the formation of ONOO-. Furthermore, we demonstrate that iNOS-mediated apoptosis of cardiac myocytes occurs in both neonatal and adult cells.

Materials and Methods

Isolation of Primary Cultures
Animals were obtained from Charles River Laboratories (Lexington, Mass), and all protocols conformed to institutional guidelines. NRVMs from 1-day-old Sprague-Dawley rats were isolated and cultured to confluence. Cardiac microendothelial cells (CMECs) from adult rat hearts were isolated and grown to subconfluence. Calcium-tolerant ventricular myocytes were then isolated from adult rat ventricular myocytes (ARVMs) and plated on top of the CMECs as a heteroculture in defined medium.

Biochemical Assays
Nitrite in the medium was measured by the Griess reaction. Intracellular cGMP concentration was measured using an enzyme immune assay kit (Cayman Chemical Co, Ann Arbor, Mich). Cell Survival by Metabolism of MTT: After a 2-hour coincubation with anhydrous MTT (Sigma), NRVMs were solubilized with dimethyl sulfoxide and Sorenson’s glycine buffer, and the OD of was measured.

Immunoblotting for iNOS Protein
Equal quantities of total protein from the supernatant of detergent-lysed NRVMs were separated by 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, which was blocked with a nonnitrosylated control reagent (Transduction Laboratories, Lexington, Ky), followed by a goat anti-mouse secondary antibody conjugated with peroxidase (Sigma) and autoradiographed using chemiluminescence (NEN Dupont, Bedford, Mass).

Northern Blotting for mRNA of Bax and Bel-2
Total RNA was isolated from NRVMs using Trizol (Gibco BRL), electrophoresed in a 1% agarose/formaldehyde gel, transferred to a transfer membrane (NEN Life Science Products, Boston, Mass), and hybridized with QuikHyb (Stratagene, La Jolla, Calif) with 32P-dCTP-labeled (ICN, Costa Mesa, Calif) cDNA probe (Random primers DNA labeling system, Gibco BRL) for Bax, GAPDH, and then Bcl-2 respectively, followed by autoradiography. The quantity of Bax and GAPDH mRNA was estimated by measurement of the integrated density of the respective digitally scanned autoradiograph band using Scion Image software.

Determination of Apoptosis
Nuclear Size Determination and Quantitation by Flow Cytometry
Ethanol-fixed NRVMs were suspended in PBS containing propidium iodide (Sigma) and RNase A (Sigma), after which 10,000 cells were analyzed by flow cytometry using a laser with an excitation wavelength of 488 nm and an emission wavelength of >600 nm and forward light scatter. The hypodiploid cells within one log of the G0/G1 peak (sub-G1 fraction) were considered apoptotic.

Hoescht Nuclear Staining
Paraformaldehyde-fixed NRVMs were stained with Hoescht 33258 and photographed under UV light.

Terminal Deoxynucleotidyl Transferase-Mediated Fluorescein-dUTP Nick End-Labeling (TUNEL) Staining
Cells were fixed with paraformaldehyde, and newly formed free ends of DNA were nick-end labeled with fluorescein-dUTP with a commercial kit (Boehringer-Mannheim) before photography using an epifluorescent microscope with excitation/emission wavelengths of 495/520 nm. In the heterocultures of ARVMs and CMECs, the cells were costained with Texas Red-X phalloidin (Molecular Probes), with excitation/emission wavelengths of 591/608 nm.

DNA Gel Electrophoresis
The supernatant of detergent-lysed NRVMs was incubated with DNase free RNase A (Sigma), then proteinase K (Sigma). Its DNA was precipitated and electrophoresed on a 2.5% agarose gel, followed by ethidium bromide staining and photography under UV light with an orange filter.

Statistical Analysis
All replicate data are expressed as mean±SEM. In experiments with comparison of two treatments, a nonpaired t test was used. In experiments with multiple treatments, ANOVA was used followed by Dunnett’s multiple comparison test. In experiments comparing different treatments for two sets of conditions, two-way ANOVA was performed. Statistical significance was achieved at a value of P<0.05 (two-tailed test).

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

NRVM Cytotoxicity by Apoptosis due to Exogenous NO or ONOO-
To determine the mechanism(s) contributing to the cytotoxicity of exogenous NO on NRVM primary cultures, either the NO donor S-nitroso-N-acetylcysteine (SNAC) or the nonnitrosylated control reagent N-acetylcysteine (NAC) was added to NRVMs in serum-free medium incubated another 48 hours. In triplicate experiments (Figure 1A), 1 mmol/L (but not 100 μmol/L) SNAC resulted in significant cell death, with cell survival only 31±23% of that of serum-starved control NRVMs at 48 hours. (ANOVA, P=0.0067; Dunnett’s multiple comparison test, serum-starved versus SNAC 1 mmol/L, P<0.01). This cell death appeared to be due to apoptosis (Figure 1B). In quadruplicate experiments, the percentage of apoptotic cells, as determined by flow cytometric analysis, was only 5.7±3.4% in serum-starved NRVMs and 7.6±3.1% and 5.1±1.8% after 48 hours of incubation with either 1 mmol/L NAC or 100 μmol/L SNAC, respectively. However, 48 hours of incubation with 1 mmol/L SNAC resulted in 33.2±5.6% apoptotic cell death (ANOVA, P=0.0002; Dunnett’s multiple comparison test, serum-starved controls versus 1 mmol/L SNAC, P<0.01). Hoechst 33258 staining of nuclei in parallel experiments showed morphological changes also suggestive of apoptosis (Figure 2).

To further examine the mechanism by which NO induced NRVM apoptosis, the related oxidant molecule ONOO- or its
phosphate-buffered saline (PBS) pH-inactivated negative control (see Materials and Methods) was incubated at increasing concentrations in serum-starved NRVM primary cultures for 48 hours. Incubation of serum-starved NRVMs for 48 hours with SNAC but not its nonnitrosylated control, NAC, resulted in decreased myocyte survival as assayed by the MTT cell respiration assay (A) and increased apoptosis as determined by flow cytometric analysis of the sub-G1 fraction after labeling with propidium iodide (B). A concentration of 1 mmol/L SNAC was associated with a significantly decreased rate of cell death (ANOVA, *P*<0.0067; Dunnett’s multiple comparison test, control vs SNAC 1 mmol/L, *P*<0.01) and a significantly increased rate of apoptosis (ANOVA, *P*<0.0002; Dunnett’s multiple comparison test, control vs 1 mmol/L SNAC, *P*<0.01).

Figure 1. The NO donor SNAC decreases NRVM survival and increases the rate of myocyte apoptosis at 48 hours. Incubation of serum-starved NRVMs for 48 hours with SNAC but not its nonnitrosylated control, NAC, resulted in decreased myocyte survival as assayed by the MTT cell respiration assay (A) and increased apoptosis as determined by flow cytometric analysis of the sub–G1 fraction after labeling with propidium iodide (B). A concentration of 1 mmol/L SNAC was associated with a significantly decreased rate of cell death (ANOVA, *P*<0.0067; Dunnett’s multiple comparison test, control vs SNAC 1 mmol/L, *P*<0.01) and a significantly increased rate of apoptosis (ANOVA, *P*<0.0002; Dunnett’s multiple comparison test, control vs 1 mmol/L SNAC, *P*<0.01).

IL-1β- and IFNγ-Induced NRVM Cytotoxicity Is Mediated by iNOS

iNOS, normally not present in untreated NRVM primary cultures, could be detected at 4 hours after the commencement of incubation with recombinant human IL-1β (rhIL-1β) 4 ng/mL and recombinant murine IFNγ (rmIFNγ) 0.05 U/mL (cytokines) in DMEM +1% ITS, supplemented with 3 mmol/L arginine and 40 μmol/L sepiapterin, and increased over time (Figure 4A). Similarly, although there was no measurable nitrite in untreated, serum-starved NRVMs, an increase in nitrite content could be detected at 6 hours, which increased with time to 28.5 μmol/L after a 24-hour incubation (Figure 4B). Coincubation of cytokine-treated NRVMs with 100 μmol/L 2-amino-5,6-dihydro-6-methyl-1H-1,3-thiazine (AMT), a specific iNOS inhibitor, 29 resulted in no detectable nitrite in the medium after 24 and 48 hours (Figure 4B).

To determine whether iNOS induction contributed to cytokine-mediated NRVM cytotoxicity, serum-starved NRVMs were treated with cytokines in the presence or absence of AMT. Incubation of serum-starved NRVMs with arginine and sepiapterin alone resulted in cell survival of 99±1% compared with serum-starved NRVMs (n=8; *P*<NS) and no significant difference in the rate of apoptosis (8.4±1.4% in serum-starved NRVMs and 10.9±1.8% in arginine- and sepiapterin-treated NRVMs [n=12; *P*=NS]). AMT alone, 100 μmol/L, had no effect on cell survival in serum-starved NRVMs after 48 hours of incubation. Survival in cytokine-treated NRVMs decreased over time to 81±2% (n=2) at 24 hours and 44±12% at 48 hours compared with serum-starved untreated NRVMs (Figure 4C). However, in the presence of 100 μmol/L AMT, cytokine treatment resulted in a significant decrease in cell survival compared with serum-starved untreated NRVMs (94±0% at 24 hours and 114±16% at 24 and 48 hours, respectively).

To verify these findings, the effects on cytokine-induced NRVM cytotoxicity and apoptosis of a nonselective NOS inhibitor, L-nitroarginine (LNA), were compared with those of the selective iNOS antagonist AMT. Serum-starved NRVMs were incubated with cytokines and either 3 mmol/L LNA or 100 μmol/L AMT for 48 hours. Neither LNA nor AMT alone was associated with any significant cell death (Figure 5A). Cytokine treatment resulted in cell survival of 62±5% (n=8) compared with that of serum-starved untreated NRVMs. NOS inhibition in cytokine-treated NRVMs completely prevented cell death at 48 hours, with cell survival, compared with untreated NRVMs of 100±3% (n=4) and 103±4% (n=4) in LNA- (3 mmol/L) and AMT- (100 μmol/L) treated NRVMs, respectively (two-way ANOVA: cytokine treatment, *P*=0.0242; NOS inhibition, *P*<0.0001; interaction, *P*<0.0001).

The percentage of apoptotic cells in serum-starved untreated NRVMs and those treated with either LNA or AMT...
alone was 5.2±1.2% (n=20), 3.5±0.7% (n=5), and 2.5±0.8% (n=3) (Figure 5B). Incubation with cytokines alone for 48 hours resulted in 29.2±4.8% apoptotic cell death (n=20 replicates), whereas parallel incubations of cytokine-treated NRVMs in the presence of LNA (3 mmol/L) or AMT (100 μmol/L) were 7.7±2.0% (n=17) and 1.9±0.3% (n=4), respectively (two-way ANOVA: cytokine treatment, \( P=0.0357 \); NOS inhibition, \( P=0.0025 \); interaction, \( P=0.0113 \)).

Further evidence of iNOS-mediated programmed cell death in NRVMs was seen in parallel experiments by nuclear staining with Hoescht 33258 (Figure 2), TUNEL staining (Figure 6), and DNA electrophoresis (Figure 7). Cytokine-treated NRVMs demonstrated morphological and biochemical evidence of apoptosis after iNOS induction.

Parallel experiments were performed comparing confluent NRVMs incubated in medium containing 10% FCS with myocytes incubated in serum-free medium. In the absence of cytokines, there were no significant differences in the extent of apoptosis in untreated cells by measurement of the sub–Gₐ fraction, TUNEL staining, or DNA electrophoresis (data not shown). Furthermore, cytokine treatment of serum-treated confluent NRVMs was associated with a similar rate of iNOS-mediated apoptotic cell death, as we observed in serum-free cells (data not shown).

iNOS-Induced Myocyte Apoptosis in ARVMs

ARVMs, in contrast to NRVMs and CMECs, produce low concentrations of NO from cytokine-induced iNOS. As a consequence, ARVM treatment with IL-1β and IFN-γ at the
concentrations used was not cytotoxic (data not shown). To determine whether a high concentration of NO from an endogenous paracrine cellular source would induce cytotoxicity in ARVMs, primary heterocultures of ARVMs and CMECs were treated with cytokines in the presence or absence of 100 μmol/L AMT for 72 hours. The cells were then fixed for TUNEL staining. As shown in Figure 8, we qualitatively demonstrated that iNOS-mediated apoptosis occurred in ARVMs within 72 hours in triplicate experiments.

iNOS-Induced Myocyte Apoptosis Is Not Mediated by cGMP

To determine whether IL-1β- and IFN-γ-induced apoptosis in NRVMs was mediated by an NO-dependent increase in cGMP, serum-starved NRVMs were incubated with cytokines, arginine, and sepiapterin in the presence or absence of the soluble guanylyl cyclase inhibitor ODQ for 48 hours. The intracellular cGMP concentration was 1.9±0.5 pmol/mg protein in serum-starved NRVMs and 3.5±0.2 pmol/mg protein in NRVMs treated with cytokines. ODQ (1 μmol/L) decreased myocyte cGMP content to 2.0±0.8 pmol/mg protein (P=NS compared with control NRVM cultures). ODQ incubation alone caused no significant decrease in cell survival but did not influence cytokine-induced cell death (Figure 9A). Cell survival in cytokine-treated NRVMs was 39±10% (n=6) of untreated, serum-starved NRVMs and 43±17% and 27±11% when coincubated with either 1 μmol/L (n=4) or 10 μmol/L (n=4) ODQ, respectively (two-way ANOVA: cytokine treatment, P<0.0001; ODQ treatment compared with cytokines, P=NS; interaction, P=NS). Similarly, the extent of apoptosis in cytokine-treated NRVMs was not decreased by coincubation with ODQ (Figure 9B). The percentage of apoptotic cells increased from 6.3±2.6% (n=9) in serum-starved, untreated NRVMs at 48 hours to 22.8±3.6% (n=6), 24.9±3.6% (n=6), and 29.9±6.9% (n=6) in cytokine-treated serum-starved NRVMs and cytokine-treated myocytes coincubated with 1 μmol/L and 10 μmol/L ODQ, respectively (two-way ANOVA: cytokine treatment, P<0.0001; ODQ treatment, P=NS; interaction, P=NS). The lack of an effect of ODQ on cytokine-in-
Serum-starved NRVMs were incubated for 24 to 48 hours, with or without cytokines, arginine, and sepiapterin, in the presence or absence of 100 μmol/L MnTBAP (Figure 10A). Cell survival in serum-starved NRVMs incubated with MnTBAP in the absence of cytokines was 96±3%, whereas cytokines decreased NRVM cell survival to 47±8% (n=4 separate experiments). In contrast, coincubation of cytokines with MnTBAP resulted in cell survival that was not significantly different from serum-starved NRVMs alone, at 114±2% (two-way ANOVA: cytokine treatment, P=0.01; MnTBAP treatment, P<0.0001; interaction, P<0.0001).

There was a small but statistically significant increase in the rate of apoptosis at 48 hours from 6.9±1.5% (n=11) in serum-starved NRVMs to 12.5±2.3% (n=8) in MnTBAP-treated NRVMs (P=0.007). However, as shown in Figure 10B, the apoptotic rate decreased from 28.0±6.6% (n=11) in cytokine-treated NRVMs to 16.4±3.8% (n=12) in NRVMs coincubated with MnTBAP (two-way ANOVA: cytokine treatment, P<0.0062; MnTBAP treatment, P=NS; interaction, P=0.0413).

In parallel experiments (Figure 7), the extent of DNA ladderin NRVMs was decreased by coincubation of cytokines with 100 μmol/L MnTBAP compared with cytokines alone. These data indicate that an agent that scavenges both superoxide and ONOO⁻ could prevent cytokine-induced toxicity by attenuating apoptotic cell death.

**Increased iNOS-Mediated Bax Expression in Cytokine-Treated NRVMs**

In triplicate experiments, the expression of Bax and Bcl2 mRNA in NRVMs was quantified 24 hours after cytokine treatment with or without coculture of 100 μmol/L AMT. Bcl-2 expression remained low at all conditions, making accurate quantification unreliable (Figure 11A). In contrast, Bax expression (Figure 11B) increased 9.5±3.4-fold 24 hours after cytokine treatment and only increased 2.2±0.4-fold in NRVMs treated with cytokines and AMT compared with untreated, serum-starved NRVMs (ANOVA, P=0.027; Dunnett’s multiple comparison test: serum-starved controls versus cytokine-treated NRVMs, P<0.05).

**Conclusions**

We have demonstrated that both an exogenous NO donor (1 mmol/L SNAC) and exogenous ONOO⁻ (100 μmol/L OONO⁻) induced predominantly programmed cell death (apoptosis) within 48 hours in serum-starved primary cultures of NRVMs. Furthermore, endogenous NO released by these cells after induction of iNOS was responsible for programmed cell death induced by the cytokine combination IL-1β and IFNγ. This iNOS-mediated cytotoxicity was not only confined to neonatal myocytes but was also induced in the adult myocyte phenotype cocultured with cardiac microvascular endothelial cells after exposure to cytokines. The mechanisms by which NO mediates apoptosis do not involve activation of guanylyl cyclase but appeared to be due predominantly to the formation of ONOO⁻ and an associated increase in the ratio of Bax to Bcl-2 expression. This confirms the recent report of Ing et al² in mice further to elucidate the mechanisms involved in the activation of apoptotic pathways.

---

**Figure 5. Inhibition of endogenous production of NO by NOS inhibitors prevents iNOS-mediated myocyte apoptosis.** Results of the incubation for 48 hours of serum-starved NRVMs with or without cytokines (Cytokines and Control, respectively) in the presence of medium alone (Baseline) or either of the NOS inhibitors LNA (3 mmol/L) or AMT (100 μmol/L) are illustrated for cell survival by MTT assay (A) (2-way ANOVA: cytokine treatment, P=0.0329; NOS inhibition, P=0.0001; interaction, P<0.0001) and for the rate of apoptosis as measured by the magnitude of the sub–G₁ fraction on flow cytometric analysis (B) (2-way ANOVA: cytokine treatment, P=0.0357; NOS inhibition, P=0.0025; interaction, P=0.0113).

**iNOS-Induced Myocyte Apoptosis Is Mediated by ONOO⁻**

As we have shown that exogenous ONOO⁻ was capable of inducing apoptosis in NRVMs and that cytokine-induced apoptosis was not mediated by cGMP, we examined the possibility that ONOO⁻ was at least in part responsible for apoptotic cell death from endogenous sources of NO and superoxide. We used the cell-permeable porphyrin analogue Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), which has been shown in previous reports to be a superoxide dismutase (SOD) mimetic and ONOO⁻ but not an NO scavenger³⁵–³⁷ with no direct effect on iNOS activity.³⁶
Figure 6. iNOS-mediated apoptotic cell death is confirmed with TUNEL staining of NRVMs after treatment with IL-1β and IFN-γ. Representative photomicrographs of reverse-phase microscopy of untreated, serum-starved confluent NRVMs (A); the corresponding fluorescein TUNEL staining of nuclei from panel A (B); reverse-phase microscopy of NRVMs 40 hours after incubation with IL-1β and IFN-γ (C); the corresponding fluorescein TUNEL staining of nuclei from panel C (D); reverse-phase microscopy of NRVMs 40 hours after incubation with IL-1β, IFN-γ, and 100 μmol/L AMT (E); the corresponding fluorescein TUNEL staining of nuclei from panel E (F); negative control fluorescein TUNEL staining excluding terminal deoxynucleotidyl transferase (see Materials and Methods) of untreated, serum-starved confluent NRVMs (G). These photomicrographs indicate the presence of newly formed free ends of DNA indicative of apoptosis in cytokine-treated NRVMs, which is prevented by inhibition of iNOS in the cells. Marker indicates 40 μm.
Despite the evidence presented in the present study suggesting a predominantly apoptotic mechanism for NO-mediated cardiac myocyte cytotoxicity, many of the assays for determination of apoptosis are not specific, making it impossible to exclude an element of necrosis in combination with apoptosis. The ratio of apoptosis to necrosis remains unclear.

There was a clear concentration threshold required to induce apoptosis after exposure to exogenous NO in these serum-starved NRVM primary cultures. Although the concentration of SNAC required to induce apoptosis might imply the need for supraphysiological concentrations of NO, Matthews et al. reported that free NO concentration in solution 3 minutes after the addition of 1 mmol/L S-nitroso-N-acetylpenicillamine (SNAP) released ≈70 nmol/L free NO. SNAP has a shorter half-life than SNAP, suggestive of a faster release and higher concentration of free NO in solution, although the concentration is unlikely to be more than doubled. In addition, Messmer and Brune demonstrated that the integral of the NO concentration over time accounted for the extent of apoptosis induced by NO donors in macrophages. Therefore, a single, short exposure to a high concentration of NO or ONOO⁻ or a combination of moderately high concentration of the same over a longer period of time, such as occurs after iNOS induction (see Figure 4), could result in programmed cell death.

Although NRVMs, like other cardiac myocyte phenotypes, do express a constitutive, calcium-dependent NOS (ie, endothelial NOS [eNOS]), it is clear that the relatively small amounts of NO generated by this enzyme would not give an adequate cumulative dose over the same period of time as iNOS and be capable of inducing apoptosis. This was suggested by the low rates of apoptosis in confluent, serum-starved NRVMs and the lack of effect of the eNOS inhibitor LNA on this baseline apoptosis. Furthermore, the choice of neonatal rather than adult rat ventricular myocyte phenotypes for the majority of the experiments reported in the present study was because of their low basal apoptotic rate, the high and sustained concentration of NO generated by iNOS after cytokine treatment, and the difficulty in performing many of the assays on ARVMs when grown in heteroculture with CMECs.

ONO⁰⁻ formation occurs after the reaction of NO with superoxide, the kinetics of which are faster than those of superoxide with SOD. Exogenous NO donors have been shown, even at low concentrations, to inhibit multiple enzymes involved in mitochondrial electron transfer, resulting in increased superoxide production and therefore increased substrate for the formation of ONOO⁻, which itself may act as an inhibitor of mitochondrial respiration. Similarly, endogenous sources of NO production in J774 cells treated with lipopolysaccharide (LPS) and IFNγ have been shown to decrease mitochondrial respiration, and the combination of ONOO⁻ and NO appeared to have additive effects. Therefore, it is likely that excessive release of NO after iNOS induction in NRVMs would result in increased superoxide and, therefore, ONOO⁻ production in the absence of other inducers of oxidative stress.

A number of studies have documented the roles of NO and/or ONOO⁻ in programmed cell death, in a time- and concentration-dependent fashion. For example, exogenous NO donors have been shown to induce apoptosis in HL-60, NA cells, murine L929, and NIH3T3 cells, and exogenous ONOO⁻ has been shown to induce apoptosis in HL-60 cells, PC12 cells, and rat thymocytes. A role for NO in IL-1β- and IFNγ-induced apoptosis has been demonstrated in mixed neuronal and glial cell cultures and in cardiac myocytes. Similarly, the combination of LPS- and IFNγ-induced apoptosis in macrophages has been shown to be mediated by NO, although some cells are resistant to NO- or ONOO⁻-mediated programmed cell death. NO and ONOO⁻ may initiate and/or regulate programmed cell death pathways at several levels. Although NO exposure to purified DNA does not cause single strand breaks even at concentrations as high as 1 mol/L, NO inactivates a DNA ligase, probably by nitrosation of a functionally critical lysine group, whereby decreasing normal DNA repair capability. In contrast, ONOO⁻ itself can initiate DNA cleavage at every
INOS-mediated apoptotic cell death is confirmed with TUNEL staining in ARVMs cocultured with CMECs within 72 hours of treatment with IL-1β and IFNγ. Representative photomicrographs of ARVMs in heteroculture with CMECs. A, Texas Red-X phalloidin–stained untreated ARVMs showing typical morphology. (CMECs can be seen staining faintly in the background.) B, Corresponding field with TUNEL staining. The rounded cell phalloidin-stained cell in panel A has positive nuclear staining. C, Texas Red-X phalloidin staining of a cytokine-treated coculture; all of the ARVMs appear to be rounding up at 72 hours. D, Corresponding TUNEL staining shows numerous apoptotic bodies within the cytosol and exterior of the ARVMs with no remaining nuclei present in the ARVMs. The CMECs do not appear to be positively stained by TUNEL. E, Texas Red-X phalloidin staining of cells treated with cytokines and 100 μmol/L L-AMT. The morphology of the ARVMs suggests a change in phenotype, but the cells are not rounded. F, Corresponding TUNEL shows no nuclear staining. Marker indicates 20 μm.
nucleotide, which is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP-ribosyl)synthetase (PARS). 27,54,55

Induction of iNOS in RAW 264.7 macrophages was shown to increase the activity of the tumor suppressor factor p53, which preceded apoptosis.48,56 Furthermore, Messmer and Brune 57 demonstrated that there was a positive correlation between the concentration of an exogenous NO donor, p53 upregulation, and the extent of DNA fragmentation over time. These authors also demonstrated the facilitatory, but not obligatory, role of p53 with NO-induced apoptosis of a p53-negative cell line, U937.

Unlike p53, increased expression of Bcl-2 has been correlated with an increase in cellular resistance to exogenous NO-induced apoptosis,25 and transfection of Bcl-2 has led to resistance to both exogenous and endogenous NO-mediated apoptosis.25,58 We demonstrated no change in Bcl-2 expression, but increased iNOS-mediated Bax expression, which placed the cell in a “proapoptotic” state.31,59

The redox state of the intracellular environment appears to influence NO-induced apoptosis, although the interactions are consistent with the complexity of NO and ONOO– chemistries.23 Significant depletion of intracellular glutathione, an important intracellular antioxidant, before incubation of J774 macrophages with LPS and IFNγ, has been shown to exacerbate NO-mediated cytotoxicity.60 Similarly, inhibition of Cu/Zn SOD activity in PC12 cells after transformation with an antisense oligonucleotide resulted in NO-mediated apoptosis.61

The pathways by which ONOO– activates cardiac myocyte apoptosis via increased Bax expression require further elucidation and comparison with other cell types. Other non-cGMP effects of NO, such as S-nitrosation of thiols, which results in modulation of critical protein functions,62 have not been excluded in the present study. MnTBAP showed attenuation without complete inhibition of iNOS-induced apoptosis.61

The relevance of NO and ONOO– generated by iNOS, in either myocytes or other cells such as microvascular endothelium and fibroblasts, to cardiac myocyte apoptosis will
only be fully appreciated by in situ experimental animal models and in humans. Although there have been several reports indicating that iNOS expression and the rate of apoptosis are increased in patients with heart failure, there have been no data to date that clearly implicate NO released by iNOS in the pathogenesis of the heart failure syndrome in humans. It appears possible that if substantial expression of iNOS occurs in cardiac failure, the actions of the NO and ONOO− generated on mechanical function, energetics, and on pathways leading to programmed cell death in cardiac myocytes may all be contributing factors to its pathophysiology and progression.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (HL52320). Margaret Arstall was the recipient of the National Heart Foundation of Australia Ralph Reader Overseas Research Fellowship 1995-1998 and the Royal Australian College of Physicians JJ Billings Overseas Research Fellowship 1995.

References


12. Simmons WW, Closs EI, Cunningham JM, Smith TW, Kelly RA. Cyto-


20. Mohr S, Stamler JS, Brune B. Posttranslational modification of glycer-


27. Ing DJ, Zang J, Dzau VJ, Webster KA, Bishopec NH. Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ Res. 1999;84:21–33.


Cytokine-Mediated Apoptosis in Cardiac Myocytes: The Role of Inducible Nitric Oxide Synthase Induction and Peroxynitrite Generation
Margaret A. Arstall, Douglas B. Sawyer, Ryuji Fukazawa and Ralph A. Kelly

Circ Res. 1999;85:829-840
doi: 10.1161/01.RES.85.9.829
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/85/9/829

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/1999/10/25/85.9.829.DC1

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/