Journal of Cellular Biochemistry 77:624–634 (2000)

L-Arginine Inhibits Apoptosis Via a NO-Dependent Mechanism in Nb2 Lymphoma Cells

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Abstract Prolactin (PRL) inhibits apoptosis and stimulates proliferation of the PRL-dependent rat Nb2 lymphoma cell line by divergent signaling pathways. Nitric oxide (NO) was recently identified as a downstream regulator of PRL action, and as an inhibitor of apoptosis in immune cells. In the present study, the role of NO in PRL-regulated Nb2 cell function was investigated. Nb2 cells expressed the endothelial nitric oxide synthase (eNOS) isoform, whereas neuronal NOS (nNOS) and inducible NOS (iNOS) mRNAs were undetectable. The eNOS mRNA was abundantly expressed in PRL-deprived, growth-arrested cells but decreased by at least 3-fold at 3–24 h following PRL treatment. Downregulation of eNOS was not accompanied by a corresponding decrease in the eNOS protein, the level of which remained constant for at least 24 h after PRL treatment. PRL had no effect on the phosphorylation state or subcellular redistribution of the eNOS enzyme, or on production of NO by Nb2 cells. However, increasing concentrations of L-arginine (NOS substrate) alone increased NO production in these cells and significantly enhanced PRL-stimulated cell proliferation. NO releasers (SNAP, DEA/NO, SIN-1) also significantly enhanced Nb2 cell proliferation in the presence of a submaximal dose of PRL (0.125 ng/ml). In the absence of PRL, the NO releasers alone promoted cell survival and maintained a viable cell density significantly higher than that of untreated PRL-deprived cells. L-arginine or the NO releaser DEA/NO alone significantly inhibited apoptosis in Nb2 cells deprived of PRL for 5 days. Expression of the anti-apoptotic gene *bcl*-2, which was stimulated within 1 h by PRL, was upregulated by L-arginine or DEA/NO alone at 2 h and 8 h, respectively. These findings suggest that NO produced by eNOS inhibits apoptosis and promotes the survival of growth-arrested Nb2 lymphoma cells via a prolactin-independent, Bcl-2-mediated pathway. J. Cell. Biochem. 77:624–634, 2000.

Key words: prolactin; nitric oxide; apoptosis; Nb2 lymphoma cells

Prolactin (PRL) is involved in a wide spectrum of physiological processes in vertebrates, including immunoregulation and lymphoid cell proliferation [reviewed in Friesen et al., 1991; Hooghe et al., 1998; Velkeniers et al., 1998]. The PRL receptor (PRLR) is expressed on human, rat, and murine lymphoid cells [Clevenger et al., 1990; Pellegrini et al., 1992], and PRL has been shown to stimulate T-cell proliferation [Clevenger et al., 1992] and to regulate T-cell differentiation [Hosokawa et al., 1996]. PRL is produced and secreted by human lymphoid tissues, thymocytes, and lymphoblastoid cell lines, indicating a paracrine or autocrine role in immune cells [DiMattia et al., 1988; Neal et al., 1992].

The mitogenic action of PRL in immune cells has been studied extensively using the rat Nb2

Grant sponsor: Medical Research Council of Canada-Burroughs Wellcome; Grant sponsor: Murray MacNeil Summer Studentship; Grant sponsor: Medical Research Council of Canada.

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Received 28 September 1999; Accepted 23 December 1999

This article published online in Wiley InterScience, April 2000.

lymphoma cell line. Nb2 cells are thymic in origin [Fleming et al., 1982], have specific, high-affinity cell surface PRLRs, and are critically dependent on PRL for growth [Gout et al., 1994]. Signal transduction by the PRLR is mediated primarily by the JAK2 tyrosine kinase-STAT (signal transducers and activators of transcription) pathway, resulting in the nuclear translocation of the STAT proteins to regulate transcription of PRL-responsive genes [reviewed in Clevenger et al., 1998; Yu-Lee et al., 1998]. In addition to its stimulatory effects on cell proliferation, PRL has been shown to promote survival of Nb2 cells by inhibition of glucocorticoid (GLU)-induced apoptosis [Fletcher-Chiappini et al., 1993; LaVoie and Witorsch, 1995; Witorsch et al., 1993]. The anti-apoptotic actions of PRL are not mimicked by agents known to inhibit GLU-induced apoptosis in thymocytes; nor are they influenced by inhibitors of PRL-stimulated cell proliferation, thereby suggesting divergent pathways for the control of Nb2 cell proliferation and apoptosis [LaVoie and Witorsch, 1995]. The anti-apoptotic actions of PRL in Nb2 cells are associated with the rapid induction of *bcl*-2 and *pim*-1 mRNAs [Krumenacker et al., 1998; Leff et al., 1996]. Overexpression of Bag-1, a Bcl-2- and Raf-1-binding protein, has also been shown to promote Nb2 cell viability and survival [Clevenger et al., 1997].

Apoptosis can be induced by a variety of cytotoxic stimuli, or simply by withdrawal of serum or growth factors which are required for normal cell survival. In pathological states, a failure to undergo apoptosis following growth factor withdrawal may lead to abnormal cell growth and malignancy. Nitric oxide (NO), which is synthesized from L-arginine by one of three known nitric oxide synthases (NOS), may act either as an endogenous activator or inhibitor of apoptosis in different cell types [reviewed in Kim et al., 1999]. NO was reported to protect L-arginine-deficient human B lymphocytes [Mannick et al., 1994] and GLU-treated mouse thymocytes from undergoing apoptosis [Fehsel et al., 1995]. More recently, nitric oxide was shown to fully protect against UVA-induced endothelial cell apoptosis by upregulation of Bcl-2 [Suschek et al., 1999]. NO has also been implicated as a downstream effector of the effects of PRL [Kumar et al., 1997; Meli et al., 1996, 1997]. However, the possible role of NO in the PRL-dependent survival and proliferation of Nb2 lymphoma cells has not previously been examined. In the present study, we examined the effects of PRL, L-arginine, and NO modulators on NOS expression, NO production, and survival of Nb2 cells. Evidence is provided that NO is involved in promoting cell survival and inhibiting apoptosis of these cells independent of PRL action.

MATERIALS AND METHODS

Rabbit anti-iNOS (NOS2) and anti-eNOS (NOS3) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-phosphoserine antibody (clone PSR-45) was from Sigma Chemical Co. (St. Louis, MO). Donkey anti-rabbit and goat anti-mouse Ig-horseradish peroxidase conjugates were from Amersham Canada, Ltd. (Oakville, Ontario, Canada). Terminal deoxynucleotide transferase (TdT) was from Promega Corp. (Madison, WI). Biotinylated deoxyadenosine triphosphate (biotin-14-dATP) and streptavidin horseradish peroxidase were from Life Technologies, Inc. (Burlington, Ontario, Canada). Aminoguanidine hemisulfate (AG; NO synthase inhibitor), S-nitroso-N-acetylpenicillamine (SNAP; NO source), diethylamine/nitric oxide complex (DEA/NO; produces controlled release of NO in solution), 3-morpholinosydnonimine HCl (SIN-1; NO donor), and carboxy-PT10 potassium (NO scavenger) were from Research Biochemicals International (Natick, MA). L-arginine, L-ornithine, paraformaldehyde, pepsin, 3-amino-9-ethyl-carbazol, and hematoxylin were from Sigma Chemical Co.

Cell Culture

Suspension cultures of the PRL-dependent rat Nb2-11C lymphoma cells were maintained in Fischer's medium for leukemic cells containing 10% fetal bovine serum (FBS) as a source of lactogens and 10% lactogen-free horse serum (HS) as previously described [Too et al., 1987]. Confluent Nb2-11C cells ($\sim 1.2 \times 10^6$ cells/ml) were growth-arrested by culturing cells in medium containing 10% HS alone and were used within 18–24 h. The L-arginine content of Fischer's medium from different suppliers varied, e.g., 18.6 mg/l L-arginine (Life Technologies) vs. 15 mg/l L-arginine (Sigma). Unless otherwise indicated, L-arginine was added to the Sigma medium to a final concentration of 18.6 mg/l.

LPS Activation of iNOS Expression In Vivo

A 230-g male rat was injected intraperitoneally with 3 mg of the endotoxin *E. coli* lipopolysaccharide (LPS), as previously described [Brown and Tepperman, 1997]. A control rat was left untreated. After 4 h, the rats were sacrificed by cervical dislocation, and the ileum and colons were removed for total RNA extraction.

RNA Extraction and Semiquantitative RT-PCR

Total RNAs were extracted from rat tissues or from Nb2-11C cells as previously described [Puissant and Houdebine, 1990] or by using RNeasy mini kits (Qiagen Inc, Mississauga, Ontario, Canada). RNA integrity was verified on a Northern gel prior to further analysis. Reverse transcription (RT) of total RNA (1 μg) was performed in a 25-μl reaction mixture containing M-MuLV reverse transcriptase (100 U; Promega), 40 pM of random hexamer pd(N)6, 200 μM of deoxynucleotide triphosphates (dNTPs), and 1.6 U RNase inhibitor. The RT reactions were incubated at 23°C for 10 min and 42°C for 60 min, and terminated at 95°C for 5 min. A 3-μl aliquot of RT reaction was used for amplification by polymerase chain reaction (PCR), which was performed in a 25-μl reaction mixture containing 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Promega) and the respective primer pair (25 pmole each/reaction). PCR amplification was carried out as follows: 94°C, 75 s; and 20–35 cycles of 94°C for 45 sec, 67°C for 45 s, and 72°C for 60 s. For each primer pair, the linear range of the PCR reaction was first determined by varying the number of PCR cycles, as previously described [Smyth et al., 1997]. RT-PCR products were electrophoresed on 1% agarose gels. Primer pairs for PCR were chosen to span at least one intron-exon splice boundary to eliminate the possibility of amplification of genomic DNA and were as follows: neural NOS (NOS1), 5′-ACG-TTT-GGG-GTT-CAG-CAG-ATC-CA-3′ and 5′-GAC-TGA-GCC-AGC-TCC-CTG-CCC-AT-3′ (506-bp product); endothelial NOS (eNOS/NOS2), 5′-CTG-CTG-CCC-GAG-ATA-TCT-TC-3′ and 5′-AAG-TAA-GTG-AGA-GCC-TGG-CGC-A-3′ (435-bp product); iNOS, 5′-AACAACAGGAACCTACCAGCTC-3′ and 5′-GATGTTGTAGCGCTGTGTGTCA-3′ (654-bp product); rat c-*myc*, 5′-GAA-GAG-ACA-CCG-CCC-ACC-ACC-AGC-3′ and 5′-TTC-TGA-GAT-GAG-TTT-GTG-CTC-3′ (527-bp product); glyceraldehyde 3-phosphate dehydrogenase, 5′-TGA-TGA-CAT-CAA-GAA-GGT-GGT-GA-3′ and 5′-TCC-TTG-GAG-GCC-ATG-TAG-GCC-AT-3′ (272-bp product); and *bcl*-2, 5′-TAT-GAT-AAC-CGG-GAG-ATC-GTG-ATG-3′ and 5′-GTG-CAG-ATG-CCG-GTT-CAG-GTA-CTC-3′ (527-bp product). The 18S primers used were from the QuantumRNA™ 18S Internal Standards Kit (Ambion, Inc., Austin, TX). PCR amplification of *bcl*-2 was performed with a slight modification; i.e., the 25-μl PCR reaction contained 4 μl of the RT reaction mixture, 200 μM dNTPs, 1.5 mM $MgCl_2$, 25 mM KCl, 2.5 U Taq DNA polymerase (Life Technologies) and 25 pmole each of the *bcl*-2-specific primer pair. PCR amplification of *bcl*-2 was performed as follows: 94°C for 60 s, and 35 cycles of 94°C for 45 s, 62°C for 45 s, and 72°C for 60 s.

Coimmunoprecipitation and Western Analysis

For coimmunoprecipitation (co-IP) experiments, Nb2 cells (~20×10^6 cells) were rinsed in phosphate-buffered saline (PBS) and resuspended in 1 ml cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM $Na_4P_2O_7 \cdot 10H_2O$) containing 1 mM Na_3VO_4, 1 mM NaF, 5 mM EDTA, 5 mM EGTA, 0.1% IGEPAL, 0.25% sodium deoxycholate, and fresh protease inhibitors (100 μg/ml of PMSF and 10 μg/ml each of antipain, leupeptin, and pepsatin). After 30 min at 4°C, the cell lysate was disrupted by passage through a 21-gauge needle, and another 10 μl of 10 mg/ml PMSF stock were added. After a further 30 min on ice, the samples were microcentrifuged at 15,000*g* for 20 min at 4°C. Anti-eNOS antibody (1 μg) was then added to the 1-ml 15,000*g* supernatant (total cell lysate) for a 1-h incubation at 4°C. Protein A/G Plus-Agarose (20 μl; Santa Cruz Biotechnologies) was added for an overnight incubation at 4°C on a rocker platform. Immunoprecipitates were precipitated at 3,000 rpm (5 min at 4°C), and the pellet washed 4 times with 1.0 ml PBS containing 1 M NaCl. After the final wash, the pellet was resuspended in 40 μl of electrophoresis sample buffer for Western analysis, as previously described [Too et al., 1998].

In subcellular fractionation experiments, Nb2 cells (20×10^6 cells per treatment) were pelleted at 200*g* for 5 min at room temperature and placed directly on ice. To each sample were added 500 μl of cold lysis buffer containing

protease inhibitors [Too et al., 1998]. The cell pellets were gently dispersed, incubated for 30 min on ice, and disrupted by gentle passaging through a 25-gauge needle. Samples were centrifuged at 800*g* for 5 min at 4°C. Cell pellets (nuclear fraction) were resuspended in 100 μl cold lysis buffer. Supernatants were ultracentrifuged at 100,000*g* for 60 min at 4°C. The 100,000*g* supernatants (cytosolic fraction) were transferred to new Eppendorf tubes, and the pellets (microsomal fraction) were resuspended in 100 μl cold lysis buffer. All samples were kept on ice, assayed for total protein concentration (Protein Assay Kit, BioRAD, Mississauga, Ontario, Canada), and immediately aliquoted for storage at −20°C until used for SDS-polyacrylamide gel electrophoresis. For Western analysis, rabbit anti-eNOS (primary antibody) and donkey anti-rabbit Ig-horseradish peroxidase conjugate (secondary antibody) were used at 0.2 μg/ml and 1:5,000, respectively. Monoclonal anti-phosphoserine (primary) and goat anti-mouse Ig-horseradish peroxidase conjugate (secondary) were used at 1:500 and 1:1,500, respectively. Immunoreactive signals were detected with Super Signal ULTRA (Pierce, Rockford, IL), as previously described [Too et al., 1998].

In Situ End-Labeling (ISEL) Assay

Apoptotic DNA fragmentation was quantified in situ as previously described [Gavrieli et al., 1992], with some modifications. Briefly, Nb2 cells were cytospun (Shandon Cytospin 2, Fisher Scientific Ltd., Nepean, Ontario, Canada) onto glass slides silinated in 2% 3-aminopropyl-triethoxysilane (Sigma) in acetone and fixed in 4% paraformaldehyde. Cells were permeabilized by digestion with pepsin (0.5%, pH 2.0) for 20 min at 37°C, and incubated in 2% H_2O_2 (Fisher Scientific Ltd.) for 10 min at room temperature to inactivate endogenous peroxidases. Following cell equilibration in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cocadylate, 1 mM cobalt chloride), DNA fragments were elongated with 12.5 pmol/μl biotin-14-dATP and 0.4 U/μl TdT enzyme in TdT buffer for 60 min at 37°C in a humidified chamber. The slides were then immersed in terminating buffer (300 mM sodium chloride, 30 mM sodium citrate, pH 8.0) for 15 min at room temperature and in 5% horse serum for 10 min. Incorporated biotin label was then detected by incubation with streptavidin horseradish peroxidase for 45 min at 37°C. Finally, the cells were stained in 3-amino-9-carbazol (AEC; 1 mg/ml) for 45 min at 37°C and counterstained with hematoxylin. Apoptotic cells were identified microscopically and quantified by counting the number of labelled nuclei in a field of at least 300 cells.

Nitric Oxide Assay

Nb2 cells were treated as previously described [Cymeryng et al., 1999], with modifications. Briefly, growth-arrested Nb2 cells were washed, resuspended in 10 mM HEPES buffer, pH 7.4, containing 5 mM glucose, 0.145 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$. $2H_2O$ and 0.5% (w/v) bovine serum albumin (HEPES-BSA buffer). Cells (0.5×10^6 cells in 0.5 ml) were aliquoted into 12×75 mm culture tubes in the presence of increasing concentrations of L-arginine, with or without PRL at 100 ng/ml. After a 37°C incubation for 210 min in 95% air-5% CO_2, the cells were pelleted at 2,000 rpm (5 min) and the supernatants were collected for NO assay; supernatants from lysed cells (23-gauge needle) gave identical results. Total nitric oxide released into the culture medium by the cells was determined using the Total Nitric Oxide Assay Kit (Assay Designs Inc., Ann Arbor, MI), following the manufacturer's instructions. Briefly, samples to be assayed were aliquoted into microtiter plates. Nitrates were reduced to nitrites by nitrate reductase in the presence of NADH. Total nitrites in the samples and in the standards (1–25-μM solutions of sodium nitrite) were detected colorimetrically with Griess reagents I and II and measured at 540–570 nm. HEPES-BSA buffer served as sample blanks.

Statistical Analysis

Analysis of variance (ANOVA) and Scheffe's F-test were performed using Abacus Concepts, Statview (Abacus Concepts, Inc., Berkeley, CA, 1992).

RESULTS

Expression of NOS Isoforms in Nb2 Cells

The neuronal (nNOS or NOS1) and endothelial (eNOS or NOS3) isoforms are expressed constitutively in many cell types, whereas iNOS (or NOS2) is inducible by bacterial endotoxins or by cytokines [Geller and Billiar, 1998]. RT-PCR was performed to identify the

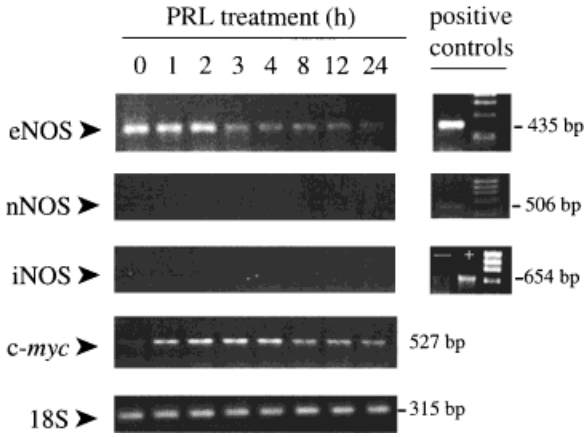

Fig. 1. Nb2 cells express eNOS mRNA. Growth-arrested Nb2 cells were treated with PRL (10 ng/ml) for the indicated times. Total RNA (1 μg/sample) prepared from the cells was used for RT-PCR, using specific primers for eNOS, iNOS, nNOS, c-*myc*, or 18S rRNA, as described in Materials and Methods. PCR products were resolved in 1% agarose gels. Positive controls for the NOS isoforms were determined using RNA prepared from normal rat heart (for eNOS), brain (for nNOS), and LPS-treated colon (for iNOS; +).

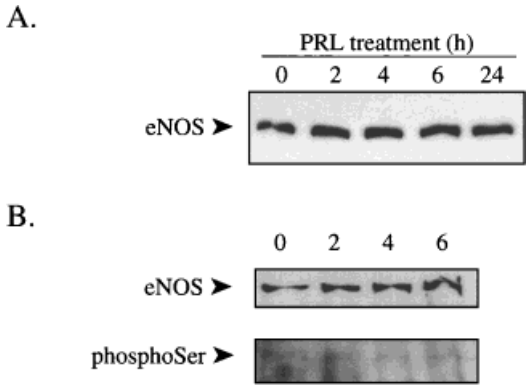

Fig. 2. Detection of eNOS protein in Nb2 cells. Growth-arrested Nb2 cells were treated with PRL (10 ng/ml) for the indicated times. **A:** Cell lysates (20 μg protein/lane) were used for SDS-PAGE in 4–20% gradient gels. Western analysis was performed with anti-eNOS antibodies (0.2 μg/ml). **B:** Total cell lysates were immunoprecipitated with anti-eNOS antibodies. Immunoprecipitates were used for SDS-PAGE in 4–20% gels. Western analysis was performed with anti-eNOS and anti-phosphoserine antibodies (1:500) (see Materials and Methods). Representative of two experiments.

NOS isoform expressed in Nb2 cells. These cells express eNOS, but not nNOS or iNOS, mRNAs (Fig. 1). The specificity of the NOS-specific primers used for RT-PCR was confirmed using RNA prepared from normal rat tissues; eNOS was expressed in rat heart and nNOS in rat brain, and iNOS was upregulated in LPS-treated rat colon as compared to controls (Fig. 1; positive controls). The expression of eNOS mRNA was elevated in growth-arrested Nb2 cells, and the level of expression remained unchanged 1 h and 2 h after PRL treatment. At 3 h, eNOS mRNA abundance decreased by about 3-fold and remained at this level for at least 24 h. Expression of the protooncogene c-*myc* is known to be upregulated in Nb2 cells in response to the mitogenic action of PRL [Fleming et al., 1985]. Figure 1 shows that the Nb2 cells in this study responded normally to PRL; c-*myc* expression was low or undetectable in quiescent cells, was elevated 1 h after PRL stimulation, peaked at 3–4 h, and remained elevated for at least 24 h.

The eNOS protein was readily detectable in Nb2 cell lysate by Western blot (Fig. 2A), and remained unchanged in growth-arrested vs. PRL-treated, proliferating cells despite the downregulation of eNOS mRNA. The iNOS protein was not detectable in the Nb2 cells regardless of cell cycle or cell density (data not shown). Western analysis of nNOS was not performed. The activity of eNOS may be regulated posttranslationally by serine phosphorylation [Dimmeler et al., 1999]. We therefore examined the effect of PRL treatment on the phosphorylation state of eNOS in Nb2 cells. Coimmunoprecipitation of eNOS, followed by Western analysis for eNOS or phosphoserine, showed no significant difference in the phosphorylation status of eNOS after PRL treatment in Nb2 cells (Fig. 2B). Hormonal stimulation of endothelial cells has been reported to cause the rapid (5–30 min) translocation of eNOS from the cell membrane to structures in the cell cytosol or to the nucleus [Feng et al., 1999; Goetz et al., 1999; Prabhakar et al., 1998]. We examined the effect of PRL on the subcellular distribution of the eNOS protein. Subcellular fractionation of Nb2 cell lysates indicated that the eNOS protein was localized predominantly in the microsomal (membrane) fraction with trace amounts present in the cytosol. PRL did not retarget the enzyme to other cellular compartments (Fig. 3). These results demonstrate that PRL has no direct effect on eNOS activity or on the subcellular redistribution of the enzyme in Nb2 cells.

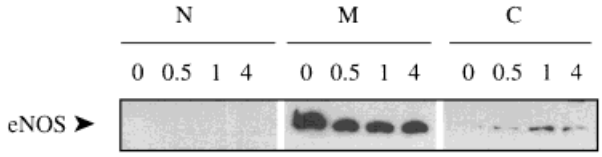

Fig. 3. Subcellular localization of eNOS protein in Nb2 cells. Growth-arrested Nb2 cells were given PRL (10 ng/ml) for the indicated times. Nuclear (N), microsomal (M), and cytosolic (C) fractions (10 μg protein/lane; see Materials and Methods) were used for Western analysis of eNOS, as described in Figure 2. Representative of two experiments.

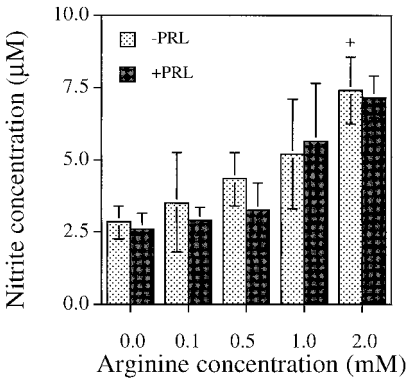

Fig. 4. L-arginine stimulates NO production. Growth-arrested Nb2 cells were resuspended in HEPES-BSA buffer. Cells (0.5×10^6 in 0.5 ml) were aliquoted into 12×75 mm culture tubes in the presence of increasing concentrations of L-arginine (±PRL at 100 ng/ml), as indicated. In each treatment, n = 3 or 4. Cells were incubated for 210 min at 37°C in 95% air-5% CO_2. Cell supernatants (2,000 rpm, 5 min) were collected for NO assay, as described in Materials and Methods. Results are a mean of three separate experiments, total n = 9 or 12. Mean ± SD; $^{+}P < 0.05$.

L-Arginine Stimulates NO Production and Enhances Nb2 Cell Growth

L-arginine, the NO precursor, is metabolized by the NO synthases to give NO and L-citrulline, or by arginase to produce urea and L-ornithine [reviewed in Geller et al., 1993]. Sustained production of high levels of NO is critically dependent on extracellular L-arginine. Growth-arrested Nb2 cells resuspended in serum- and L-arginine-free medium produced low, but measurable amounts of NO; the addition of L-arginine increased NO production in a dose-dependent manner (Fig. 4). NO production at 2.0 mM arginine was significantly higher ($P < 0.05$) than in controls with no arginine. However, PRL (up to 100 ng/ml) had no significant effect on NO production by these cells (Fig. 4).

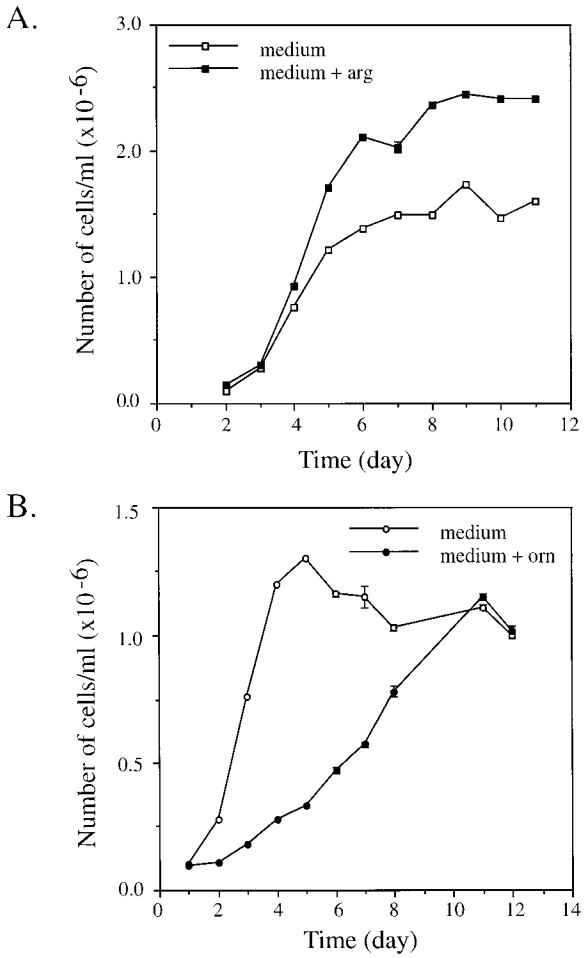

Fig. 5. L-arginine, but not L-ornithine, enhances Nb2 cell growth. Growth-arrested Nb2 cells were diluted to a cell density of ~0.1×10^6 cells/ml in 10% FBS-10% HS Fischer's medium for leukemic cells (18.6 mg/l L-arginine; Life Technologies, Inc.) (□, ○), or in the same medium to which was added (**A**) L-arginine to a final concentration of 75 mg/L (■), or (**B**) L-ornithine to a final concentration of 54 mg/l (●). Cell counts were determined daily with a Coulter (Hialeah, FL) counter. Mean ± SD (n = 3); representative of two experiments. Absence of error bars indicates small SD.

We next examined the effect of L-arginine on Nb2 cell proliferation. In Fischer's medium for leukemic cells, which contains 15.0 mg/l (~0.07 mM) L-arginine (Sigma), Nb2 cell cultures became confluent at a cell density of ~1.3×10^6 cells/ml (data not shown). However, when grown in Fischer's medium from Life Technology, Inc., which contains 18.6 mg/l (~0.09 mM) L-arginine, the cells became confluent at a higher cell density of ~1.8×10^6 cells/ml (Fig. 5A). Addition of L-arginine in this medium to a final concentration of 75 mg/l (~0.36 mM) increased the confluent cell density to ~2.5×10^6

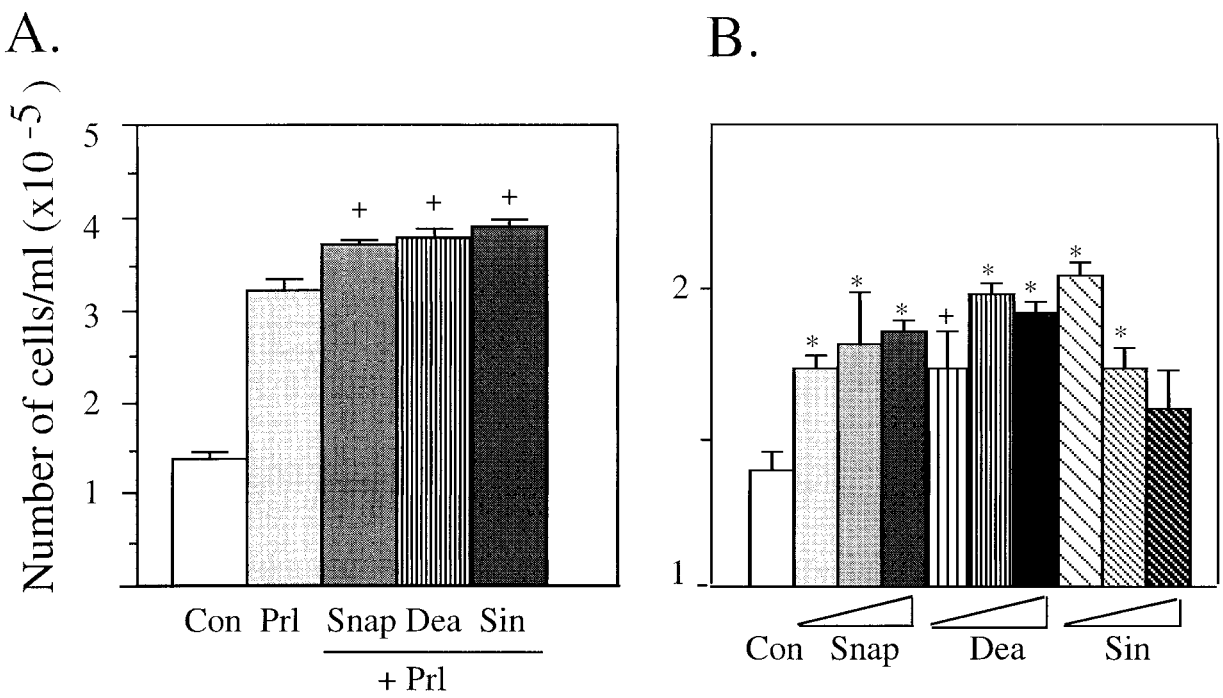

Fig. 6. NO releasers stimulate Nb2 cell growth. Growth-arrested Nb2 cells were diluted to ~0.1×10^6 cells/ml in 10% HS-Fischer's medium (Life Technologies) to which was added (**A**) a submaximal dose of PRL (0.125 ng/ml) alone or in combination with SNAP (70 μM), DEA (0.625 mM), or SIN (9.3 μM), or (**B**) increasing concentrations of SNAP alone (0.07 mM, 2 mM, 4 mM), or DEA alone (0.625 mM, 5 mM, 10 mM), or SIN alone (9.3 μM, 27.8 μM, 250 μM). Cell number was determined with a Coulter counter on day 3. Mean ± SD, n = 6; $^{+}P < 0.05$; $^{*}P < 0.01$. Representative of two experiments.

cells/ml (Fig. 5A). The stimulatory effect of L-arginine on Nb2 cell growth was not attributable to the arginase pathway, since additions of L-ornithine (arginase substrate) did not enhance cell growth (Fig. 5B).

Effect of NO Modulators on Nb2 Cell Growth

Biological roles for NO in mammalian systems have been established by manipulation of cellular NO levels by compounds that act as NO releasers or NO scavengers [Maragos et al., 1993] or as competitive NOS inhibitors [Knowles and Moncada, 1994; Misko et al., 1993]. The effect of NO releasers on Nb2 cell growth was evaluated in the presence or absence of a submaximal dose of PRL (0.125 ng/ml). As shown in Figure 6A, Nb2 cell growth in response to PRL was significantly enhanced by the NO releasers SNAP, DEA/NO, and SIN-1. Interestingly, in the absence of PRL, the NO releasers alone were able to promote cell survival and to maintain a viable cell density significantly higher than that of untreated PRL-deprived cells (Fig. 6B).

The NOS inhibitor aminoguanidine (AG) and the NO scavenger carboxy-PT10 both inhibited PRL-stimulated Nb2 cell proliferation (IC_{50} of AG = ~7 mM, IC_{50} of PT1O = ~115 μM; data not shown). The high concentration of AG required is consistent with its relative selectivity for the various NOS isoforms (iNOS > nNOS > eNOS) [Misko et al., 1993].

L-Arginine or NO Releasers Alone Stimulate *bcl-2* Expression and Inhibit Apoptosis

When Nb2 cells were growth arrested by PRL deprivation in Fischer's medium (Life Technologies; 18.6 mg/l L-arginine), ~67% of the cells were nonapoptotic after 5 days (Fig. 7, control). However, the addition of either L-arginine (up to 75 mg/l) or the NO releaser DEA/NO (0.625 mM) markedly increased cell survival; 83% and 87% of the cells were nonapoptotic after 5 days, respectively. Submaximal doses of PRL (0.125 ng/ml) also promoted Nb2 cell survival, increasing the percentage of nonapoptotic cells to ~82% on day 4 and ~90% on day 6 (data not shown).

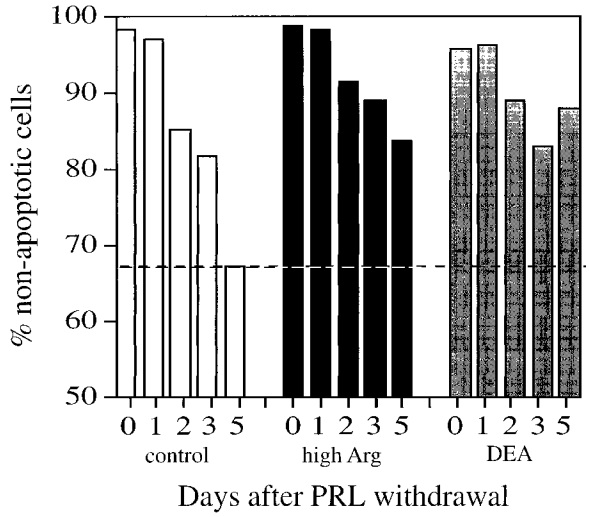

Fig. 7. L-arginine or DEA/NO promotes Nb2 cell survival. Nb2 cells were growth-arrested in 10% HS-Fischer's medium containing 18.6 mg/l L-arginine (low arginine; Control) or the same medium to which was added either L-arginine to a final concentration of 75 mg/l (high Arg) or 0.625 mM DEA (low arginine; DEA). Cells were collected for ISEL analysis on specified days as described in Materials and Methods. The results, which are representative of three independent experiments, depict the percentage of apoptotic cells determined visually by counting the number of labeled nuclei in a minimum field of 300 cells per slide.

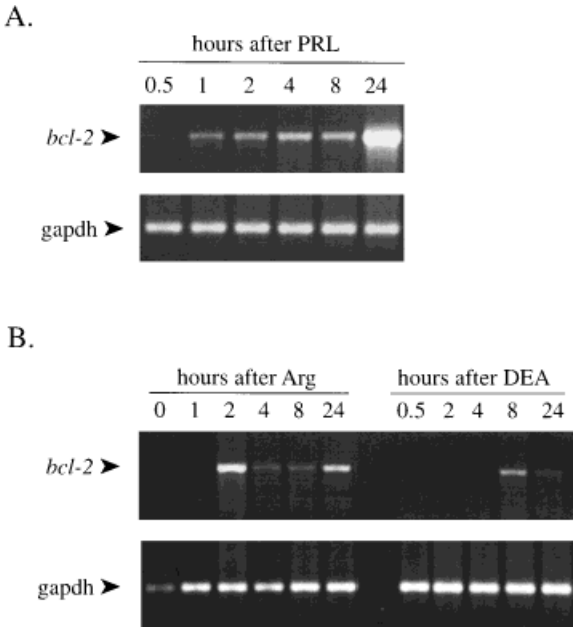

Fig. 8. PRL, Arg, or DEA/NO stimulates *bcl*-2 mRNA expression. Nb2 cells were growth-arrested for 18 h by lactogen deprivation in 10% HS-Fischer's medium containing 18.6 mg/l arginine. Cultures were harvested at the indicated times after treatment with (**A**) 10 ng/ml PRL, (**B**) L-arginine (75 ng/ml), or (**B**) DEA/NO (0.625 mM). Total RNA (1 μg/ml) was used for RT-PCR to amplify *bcl*-2 and glyceraldehyde 3-phosphate dehydrogenase (gapdh) mRNA, as described in Materials and Methods.

PRL has previously been shown to protect Nb2 cells from undergoing apoptosis by upregulating the expression of the anti-apoptotic gene *bcl-2* [Clevenger et al., 1997; Krumenacker et al., 1998; Leff et al., 1996]. As shown in Figure 8A, PRL rapidly stimulated *bcl*-2 mRNA expression within 1 h in growth-arrested Nb2 cells. Interestingly, *bcl*-2 expression in PRL-deprived cells was upregulated by L-arginine (75 mg/ml) or DEA/NO (0.625 mM) alone, but at 2 h and 8 h of treatment, respectively (Fig. 8B). The prolonged expression of *bcl*-2 24 h after L-arginine or DEA/NO treatment suggests a protective role of these two agents in the survival of PRL-deprived Nb2 cells.

DISCUSSION

There are three known isoforms of nitric oxide synthase [Geller and Billiar, 1998]. The constitutive isoforms in neurons (nNOS) and endothelium (eNOS) are Ca^{2+}/calmodulin-dependent and rapidly release small amounts of NO in response to increases in intracellular calcium. In contrast, iNOS (NOS2), which is expressed in many cell types, is Ca^{2+}/calmodulin-independent and produces larger and sustained quantities of NO in cells stimulated by bacterial endotoxins or by cytokines. About 15% of thymocytes isolated from rat thymus constitutively expressed the eNOS isoform [Cruz et al., 1998]. The PRL-dependent rat Nb2 cells are pre-T in origin [Fleming et al., 1982], and our present studies showed the exclusive expression of eNOS in growth-arrested Nb2 cells. This level of eNOS expression remained constant for up to 2 h after PRL treatment (G_0/G_1 phase of the cell cycle), but it then decreased by at least 3-fold at 3–24 h, at which time the cells were fully committed into the cell cycle. Our studies showed that PRL downregulation of the eNOS mRNA was not accompanied by a corresponding decrease in the eNOS protein level in Nb2 cells, suggesting that the eNOS protein may have a longer half-life than its mRNA. Downregulation of eNOS mRNA, but not the protein, has also been reported in human lymphocytes as well as T and B lymphoid cell lines upon cellular activation with

tumor-promoting phorbol ester (PMA), concanavalin A (ConA), or IL-4 [Reiling et al., 1996]. In fact, the eNOS protein level remained unchanged in T cells incubated for 15 h with PMA and ConA [Reiling et al., 1996]. The eNOS mRNA in endothelial cells was reported to be very stable under basal conditions, with a half-life of 48 h. However, tumor necrosis factor-α was shown to downregulate eNOS mRNA by increasing its rate of degradation, and thereby shortening its half-life to 3 h [Yoshizumi et al., 1993]. It is not known whether PRL directly increases the degradation of the eNOS mRNA in Nb2 cells.

Although PRL had no effect on the steady-state levels of the eNOS protein, there is a possibility that it may affect enzyme activity. Activation of eNOS has been shown to be mediated by serine/threonine phosphorylation, leading to increased NO production in endothelial cells [Dimmeler et al., 1999]. Hormonal stimulation has also been shown to retarget the eNOS protein, localized predominantly at the cell membrane in resting endothelial cells, to other subcellular compartments. For example, a significant amount of the protein (≥50% to all) translocated within 5–30 min following stimulation by bradykinin to structures in the cell cytosol [Prabhakar et al., 1998], by estrogen to intracellular sites close to the nucleus [Goetz et al., 1999], or by VEGF to the nucleus [Feng et al., 1999]. Our studies showed that PRL had no immediate effect on the serine phosphorylation of eNOS or on subcellular retargetting of the enzyme. Furthermore, although NO production by Nb2 cells increased with increasing concentrations of L-arginine, PRL (up to 100 ng/ml) had no significant effect on NO production by these cells. Therefore, in growth-arrested Nb2 cells, expression of eNOS may be elevated to generate NO for the promotion of cell survival during this critical stage of PRL deprivation. Upon the addition of PRL, this acute requirement for NO is relieved and is accompanied by a decrease in eNOS expression. The mechanism involved in the upregulation of eNOS during PRL deprivaton is as yet unknown.

Nitric oxide is volatile but is rapidly oxidized to two stable breakdown products, nitrate and nitrite, which can be detected by colorimetric means. We had previously attempted to measure the nitrite produced by Nb2 cells using Greiss reagent, but the assay was not sufficiently sensitive. In the present study, nitrates were reduced to nitrites and total nitrite concentration was determined. The level of NO produced by the Nb2 cells was low (5×10^5 cells produced ~7.0 μM nitrite in 210 min at 2 mM L-arginine), but comparable to levels reported for rat adrenal cells (10^5 cells produced ~5 μM nitrite in 210 min in 2.5 mM L-arginine) [Cymeryng et al., 1999]. High levels of NO produced by iNOS are known to induce apoptotic cell death. However, low levels of NO have been shown to protect B and T lymphocytes from undergoing apoptosis [Fehsel et al., 1995; Mannick et al., 1994]. Our studies showed that L-arginine (NO substrate) alone increased NO production in Nb2 cells. Furthermore, Nb2 cell survival/growth was enhanced by NO donors (SNAP, SIN-1) or by NO releaser (DEA/NO), either in the absence of PRL or in the presence of a submaximal dose of the hormone. ISEL studies showed that an increase in L-arginine concentration (from 18.6 mg/l to 75 mg/l) or the addition of the NO releaser, DEA/NO, alone dramatically decreased the percentage of apoptotic Nb2 cells during 5 days of PRL deprivation. Therefore, in the absence of PRL, NO promotes Nb2 cell survival by acting as an anti-apoptotic agent. In the presence of submaximal doses of PRL, NO enhances cell growth in response to this mitogen.

Activation of JAK2 tyrosine kinase is a first step in the mitogenic action of PRL in Nb2 cells [Yu-Lee et al., 1998], and NO as well as other thiol oxidants have been reported to inhibit the autokinase activity of rat JAK2 in vitro [Duhe et al., 1998]. Furthermore, pretreatment of quiescent mouse Ba/F3 cells with DEA/NO (in the mM range) was shown to inhibit IL-3-triggered in vivo activation of Jak2, and this correlated with inhibition of cell proliferation. Our finding of a protective action of NO in Nb2 cells does not necessarily contradict these reports. It has been suggested that the effects of cellular NO on the JAK-STAT pathway may be reversible [Bingisser et al., 1998] and may be determined by absolute levels produced in different cell types [reviewed in Kroncke et al., 1997].

The mechanism by which NO rescues B cells from antigen-induced apoptosis, without promoting a proliferative response, is believed to involve sustained levels of Bcl-2 [Genaro et al., 1995]. Endogenous or exogenously supplied NO has also been shown to fully protect against UVA-induced apoptosis by upregula-

tion of Bcl-2 in keratinocytes [Suschek et al., 1999]. Apoptotic signaling cascades converge in the activation of caspases, and it has been suggested that NO may inhibit caspase-3 activity by S-nitrosation and oxidation of critical thiol groups [Mohr et al., 1997]. The anti-apoptotic action of PRL in Nb2 cells is associated with the expression of apoptosis-related genes, including *bcl*-2, *pim*-1, and *bag*-1 [Clevenger et al., 1997; Krumenacker et al., 1998; Leff et al., 1996]. The present studies confirmed that PRL rapidly (1 h) stimulated *bcl*-2 expression in Nb2 cells. However, *bcl*-2 expression was also stimulated by L-arginine or DEA/NO alone at 2 h and 8 h, respectively.

This study showed that Nb2 cells express exclusively the eNOS isoform. Expression of eNOS in growth-arrested cells was downregulated upon treatment with PRL and was accompanied by PRL stimulation of *bcl*-2 expression. However, PRL alone did not alter the phosphorylation status of the eNOS protein, had no effect on eNOS translocation, and did not stimulate NO production in these cells. In contrast, L-arginine and/or DEA/NO alone increased NO production, increased expression of *bcl*-2, and inhibited Nb2 cell apoptosis. In conclusion, this study suggests that the anti-apoptotic action of PRL in Nb2 cells was not mediated directly by NO. However, NO produced *in*dependently of PRL promoted the survival of Nb2 cells, and raises the possibility that endogenous production of low levels of NO may play a role in the survival and progression of lymphoid tumors.

ACKNOWLEDGMENTS

We thank Dr. Roy Duncan (Department of Microbiology and Immunology, Dalhousie University) for advice on rat LPS treatment. The technical assistance of Ms. Shirley Sangster is gratefully acknowledged. F.D. received Medical Research Council of Canada (MRC)-Burroughs Wellcome and Murray MacNeil Summer Studentships. C.K.L.T. is a Scholar of the MRC. This work was supported by the MRC (to C.K.L.T.).

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