Investigation of Intracellular Signals Mediating the Anti-Apoptotic Action of Prolactin in Nb2 Lymphoma Cells (43901)

HOLLY A. LAVOIE[1] AND RAPHAEL J. WITORSCH[2]

Department of Physiology, School of Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Abstract. **Studies were undertaken to identify intracellular mediators of prolactin inhibition of glucocorticoid-induced apoptosis in Nb2 lymphoma cells. A short-term assay was implemented that quantitates fragmented DNA released from the genome by reaction with diphenylamine. Induction and inhibition of internucleosomal DNA cleavage (indicative of apoptosis) was verified by agarose gel electrophoresis of extracted cellular DNA. Synchronized Nb2 cells (G_0/G_1) exhibited increased DNA fragmentation after 4-hr incubation with dexamethasone (DEX) (25–100 n*M*) which was inhibited by ovine prolactin (oPRL) (0.1–1 ng/ml), the glucocorticoid receptor antagonist, RU486 (500 n*M*), and the nuclease inhibitor, aurintricarboxylic acid (100 μ*M*). Signals previously implicated in prolactin induction of mitogenesis in Nb2 cells were investigated for their role in prolactin inhibition of apoptosis including: protein kinase C activation, arachidonic acid metabolism, polyamine production, tyrosine phosphorylation, and extracellular calcium. Protein kinase C agonists, phorbol-12-myristate-13-acetate, and 1,2-dioctanoyl-*sn*-glycerol, ± the calcium ionophore, A23187 (200 nM), did not mimic oPRL inhibition of DEX-induced DNA fragmentation. Protein kinase C inhibitors, gossypol and quercetin, did not block prolactin action. Arachidonic acid did not mimic prolactin protection against DEX-induced DNA fragmentation. Inhibitors of arachidonic acid metabolism, 5,8,11,14-eicosatetraynoic acid, nordihydroguaiaretic acid, and indomethacin did not block prolactin action. The polyamine, spermine, inhibited DEX-induced DNA fragmentation at 1.5 to 2.5 m*M*. However, inhibition of polyamine synthesis with α-difluoromethyl ornithine or methylglyoxal *bis*(guanylhydrazone) did not inhibit prolactin action. Prolactin action was not blocked by inhibitors of tyrosine kinase activation, genistein and tyrphostin-47. On the other hand, pervanadate, a potent tyrosine phosphatase inhibitor, consistently inhibited DEX-induced DNA fragmentation. Prolactin action and DEX-induced apoptosis both occurred in calcium-free PBS. In summary, protein kinase C activation and eicosanoid production do not appear to mediate this prolactin action. Although spermine could block DNA fragmentation, blockade of the polyamine cascade did not inhibit prolactin action, suggesting that polyamines do not mediate this prolactin effect. While inhibitors of tyrosine kinase activation did not block prolactin action, tyrosine phosphatase inhibition in the presence of basal tyrosine kinase activity mimicked prolactin action, suggesting tyrosine phosphorylation participation in the anti-apoptotic effect. Extracellular calcium was not required for prolactin or DEX action.** [P.S.E.B.M. 1995, Vol 209]

[1] Current address: Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Virginia School of Medicine, Box 202, Charlottesville, VA 22908.

[2]To whom requests for reprints should be addressed at Department of Physiology, School of Medicine, Box 980551 Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0551.

Received October 21, 1994. [P.S.E.B.M. 1995, Vol 209]
Accepted January 30, 1995.

0037-9727/95/2093-0257$10.50/0

The Nb2 lymphoma cell line is a pre-T-cell line derived from a lymph node tumor in an estrogen-treated male rat (1). Nb2 cells proliferate in response to prolactin (PRL) and other lactogenic hormones which act through the mutant, intermediate form of the prolactin receptor present in these cells (2, 3). Other Nb2 cell mitogens include cytokines, interleukin-2 (IL-2) and IL-7 (4, 5). The high sensitivity of the Nb2 cell to prolactin-induced mitogenesis have made it a model system for studying prolactin mechanism of action.

Since Nb2 cells are phenotypically similar to the immature thymocyte (6), a glucocorticoid sensitive cell type, studies were undertaken to investigate the interaction of two distinctly different immunomodulatory hormones, the protein, prolactin, and the glucocortioid, dexamethasone (DEX). We previously reported two distinct effects of glucocorticoids in Nb2 cells. DEX is anti-proliferative to Nb2 cells incubated in the presence of prolactin. In the absence of prolactin, DEX is cytolytic to Nb2 cells within 12–24 hr (7). This DEX-induced cytolysis is characteristic of apoptosis as reflected by the classical pattern of internucleosomal cleavage of DNA (8, 9). Prolactin and other lactogenic hormones were effective in inhibiting DEX-induced apoptosis in a concentration-dependent manner. The observation that prolactin-treated cells do not proliferate in the presence of DEX, yet fail to undergo the apoptotic process, suggest that the mechanism of prolactin-inhibition of apoptosis diverges from prolactin-induced mitogenesis.

The anti-apoptotic action of prolactin is a novel effect of this hormone in Nb2 cells and inhibition of apoptosis may serve as a definitive endpoint in studying prolactin action in these cells. As noted previously (7), other laboratories have reported that the peptidic mitogens, erythropoietin and IL-2, inhibit apoptosis in cultured hemopoietic cells. These ligands happen to be members of the same receptor family as the prolactin receptor (10). Various mediators of prolactin-induced mitogenesis in Nb2 cells have been implicated including protein kinase C (11, 12), phospholipase activity and eicosanoid production (13–15), calcium (16–18), polyamines (19–22), serine/threonine phosphorylation (23, 24), and tyrosine phosphorylation (3, 25–29).

Protein kinase C activation has been implicated as an inhibitor of glucocorticoid-induced apoptosis in thymocytes (30). Polyamines have also been shown to protect thymocytes against glucocorticoid-induced fragmentation of DNA (31). Glucocorticoid inhibitory effects in some immune cells occur at the level of phospholipase A2 and subsequent eicosanoid synthesis (32). Thus, there are several potential sites at which prolactin inhibition of apoptosis might occur.

The purpose of this investigation was to determine what signal(s) may be responsible the anti-apoptotic effect of prolactin. Since this is the first investigation of this novel prolactin action, agonists and antagonists of signaling pathways previously implicated in prolactin action were examined. This study entailed implementing a modified short-term assay described for thymocytes which measures DNA fragmentation as a quantitative measure of apoptosis. Preliminary reports of these findings have appeared in abstract form (33, 34).

Materials and Methods

Hormones and Culture Reagents. Ovine prolactin (oPRL S-15) was provided as a gift by the National Hormone and Pituitary Program. Dexamethasone (1,4-pregnadiene-9-fluor-16α-methyl-11β,17α,21-triol-3,20-dione) was obtained from Sigma Chemical Co. (St. Louis, MO). RU 486 (11-β-[-4-dimethylaminophenyl]-17β-hydroxy-17α-(prop-1-ynyl)estra-4,9-dien-3-one) was provided as a gift by Roussel Uclaf (Paris, France). All signal agonists and inhibitors were obtained from Sigma with the exception of phorbol-12-myristate-13-acetate (TPA) and α-difluoromethyl ornithine (DFMO). TPA was obtained from Calbiochem (La Jolla, CA). DFMO was provided as a gift by Merrell Dow (Cincinnati, OH). All components for cell culture medium were purchased from Gibco BRL, Life Technologies (Grand Island, NY).

Maintenance of Nb2 Lymphoma Cells. Nb2 cells were obtained from two sources, Dr. Joseph Liberti, Department of Biochemistry and Molecular Biology, Medical College of Virginia, and Dr. Peter Gout, Cancer Control Agency of British Columbia. Nb2 U17 cells provided by Dr. Gout represent the parental prolactin-dependent wild-type (35) and were used for the majority of the experiments described herein. Nb2 cells from parental stocks were resurrected from storage in liquid nitrogen. Cells were maintained in vented tissue culture flasks in growth medium composed of Fischer's medium supplemented with 10% horse serum, 10% new born calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 m*M* 2-mercaptoethanol, pH 7.4, in a water-saturated atmosphere of 5% CO_2 and 95% room air at 38°C (standard atmospheric conditions) (7). All assay incubations described below were conducted in a chemically defined, serum-free medium (denoted SYN) previously adapted for Nb2 cells (36). SYN (pH 7.4) was prepared by supplementing Fischer's medium with final concentrations of the following: 0.1 m*M* 2-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin, 0.15% (wt/vol) essentially fatty acid free bovine serum albumin (BSA), 4 μg/ml linoleic acid, 1 m*M* sodium pyruvate, 12 μg/ml transferrin, 1.5 ng/ml selenium, 1× vitamins (diluted from a commercial stock of 100×), 0.33× amino acids (diluted from a commercial stock of 50×), 100 μ*M* spermidine, 0.5 m*M* calcium chloride. A slight modification used in this study was the addition of 10 m*M* HEPES instead of 15 m*M* HEPES in the original formula.

Acute Apoptotic Assay and Quantitative Evaluation of DNA Fragmentation. A short-term assay was designed using syncrhonized cells (predominantly in G_0/G_1) and an estimate of DNA fragmentation (i.e., DNA released from the genome) following a 4-hr incubation in SYN. The viability of cells was assessed by trypan blue exclusion and cultures with a minimum

of 90% live cells were used for apoptotic studies. Cell counts were performed prior to plating and following the 4-hr incubation using a Coulter counter as described previously (7). At the beginning of each experiment, log phase cells were removed from their growth medium, washed by centrifugation of cells at 300*g* for 7 min at 6°C, resuspended in unsupplemented Fisher's medium, recentrifuged, and finally resuspended in SYN at a concentration of 5×10^5 cells/ml. These cultures were then incubated for 22–24 hr to achieve synchronization. Cells were then washed as described above and resuspended in SYN and plated at a final concentration of approximately 2×10^6 cells/ml in final volumes of 3 ml in 6-well culture plates for the assay. Ovine prolactin (initially dissolved in 0.01 *N* NaOH) and other water soluble agents were diluted from frozen or freshly prepared stocks in SYN immediately prior to plating. Dexamethasone and other agents were dissolved in DMSO or 100% ethanol (ETOH) and were added to a final concentration of 0.25% DMSO and/or ETOH (a concentration that did not significantly affect DNA fragmentation or viability). Appropriate controls contained the same final concentrations of DMSO and/or ETOH as treatments. Cells exposed to the reagents in question and appropriate controls were incubated under standard atmospheric conditions for the desired time. The concentration ranges of signaling pathway agonists or antagonists used in these experiments were based upon previously reported effective concentrations and pilot experiments to determine minimal toxicity of these agents, as determined by cell viability or DNA fragmentation. At the end of the incubation time, approximately 3×10^6 cells of each treatment (1.5 ml) were pelleted for 10 minutes at 300*g*, 6°C and the supernatant media discarded. For quantitation of DNA fragmentation a protocol previously adapted for the Nb2 cell was used (37). Cells were lysed with 1.5 ml of a lysing buffer containing: 5 m*M* Tris, 5 m*M* EDTA, and 0.5% Triton X100, pH 7.4, on ice for 20 min with periodic vortexing. Cell lysates were centrifuged at 13,000*g* for 15 min to separate low molecular weight DNA (supernatant DNA) from high molecular weight DNA attached to the nuclear matrix (pellet DNA). Samples were allowed to react overnight with diphenylamine reagent (4% diphenylamine and 0.01% paraldehyde in acetic acid) (38), extracted with amyl acetate, and read with a spectrophotometer at 600 nm. DNA standards were prepared with each experiment to determine the OD/μg DNA. The amount of DNA in the supernatant (i.e., DNA released from the genome) normalized per 1×10^6 cells was used as the determinant of DNA fragmentation.

Evaluation of DNA Fragmentation by Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed to verify the "ladder" pattern of internucleosomal DNA cleavage associated with apoptosis. Synchronized Nb2 cells were washed and resuspended in SYN media at a final concentration of $1.5–2.0 \times 10^6$ cells/ml. Ten-milliliter volumes of cell culture with appropriate hormones, drugs and/or vehicles for each treatment were incubated in 25 cm^2 tissue culture flasks under standard environmental conditions for the desired time. Cells were pelleted by centrifugation (300*g* for 10 min at 6°C) and processed for DNA analysis by agarose gel electrophoresis as described previously (7).

Cell Cycle Analysis. The cell cycle distribution of Nb2 cells removed from growth medium and incubated in serum-free medium was analyzed to determine the time period for synchronization defined as the accumulation of cells in G_0/G_1. Cells from log phase cultures were washed and resuspended in 50 ml of SYN media at a concentration of 0.4×10^6 cells/ml. Cells were incubated and 3.5-ml aliquots removed at the desired time. One million cells were pelleted, washed and resuspended in 1 ml of propidium iodide fluorochrome stain diluted 1:10 (3.8 m*M* NaCitrate, 0.05 mg/ml propidium iodide, 0.1% Triton X100, 7 Kunits/ml ribonuclease B). Samples were filtered through a 37-μm mesh prior to analysis. Cell cycle analysis based on DNA content was performed using a Coulter Epics 753 by the Massey Cancer Center Flow Cytometry Facility (Medical College of Virginia, Richmond, VA).

Calcium-Free Modified Dulbecco's Phosphate Buffered Saline. To examine the role of extracellular calcium in prolactin and dexamethasone action, cells were assayed in calcium-free medium using a modified version of Dulbecco's phosphate buffered saline (PBS) (39). PBS was made to the final concentrations in sterile plastic bottles as follows: 2.7 m*M* KCl, 1.1 m*M* KH_2PO_4, 138 m*M* NaCl, 8.1 m*M* Na_2HPO_4, 5.6 m*M* D-glucose, 0.33 m*M* sodium pyruvate, and 0.5 m*M* $MgCl_2-2H_2O$. In addition, 1 mg/ml BSA containing linoleic acid was added to prevent cells from sticking to surfaces of the assay plates. Addition of 0.1 m*M* EGTA to this PBS reduced Ca^{2+} to essentially zero (between 10^{-9} and 10^{-11} *M* Ca^{2+}) as measured by calcium electrode. Experiments with "calcium-free" medium used the above PBS without added calcium and contained 0.1 m*M* EGTA, pH 7.2. Complete PBS contained 0.9 m*M* $CaCl_2$ and the EGTA was omitted. Cells were washed in their respective incubation medium prior to plating. Cells had higher basal DNA fragmentation in both complete and calcium-free PBS than that typically observed with SYN.

Statistics. Data from individual experiments conducted on different days were used to generate means and SE. Treatments were compared statistically by

analysis of variance and Duncan's multiple range analysis, as reported previously (7). All statistics were performed using a statistical software package (SPSS Release 4.1).

Results

Validation of Acute Apoptotic Assay. DNA content was measured by flow cytometry in Nb2 cells collected following 0-, 12-, 16-, 20-, and 24-hr incubation in serum-free medium. Flow cytometry profiles showed a time-dependent accumulation of cells in G_0/G_1. Cells from log phase cultures (0 hr) showed 54% in G_0/G_1, 35% in S, and 11% in G_2/M of the cell cycle. Twenty-four-hour cultures in serum-free medium revealed the largest number of synchronized cells with 91% in G_0/G_1, 6% in S, and 3% in G_2/M of the cell cycle. The accumulation of cells in G_0/G_1 showed no decrease in cell viability and a greater than 2-fold increase in cell number at 24 hr as cells moved through the cell cycle. Based on these results, a 22- to 24-hr preincubation period was chosen to synchronize cells.

Figure 1A shows that dexamethasone produced a concentration-dependent increase in supernatant or fragmented DNA (i.e., DNA released from the genome). A statistically significant increase in supernatant DNA was demonstrable at 25 and 100 n*M* dexamethasone. DEX (100 n*M*) usually produced a 2-fold increase in DNA in the supernatant at 4 hr. The effect of ovine prolactin on 100 n*M* DEX-induced DNA fragmentation is also shown in Fig. 1A. Prolactin caused a concentration-dependent inhibition of DEX-induced DNA fragmentation with significant inhibition at 100 pg/ml and 1 ng/ml oPRL. Since 1 ng/ml oPRL was the lowest concentration of prolactin that consistently inhibited most or all DEX-induced DNA fragmentation, this concentration was used in the majority of studies hereafter. Dexamethasone-induced DNA fragmentation could also be blocked by the glucocorticoid receptor antagonist, RU486 (500 n*M*) and the nonspecific nuclease inhibitor, aurintricarboxylic acid (100 μ*M*) (AT). RU486 and AT in the absence of dexamethasone did not affect DNA fragmentation (data not shown).

Figure 1B shows an ethidium-bromide stained agarose gel of DNA extracted from cells following a 4-hr incubation with treatments. This gel demonstrates that DNA from Nb2 cells treated with 100 nM DEX displays the characteristic "ladder" pattern of DNA fragmentation indicative of apoptosis. This laddering of DNA is inhibited by 1 ng/ml oPRL, 500 n*M* RU486, and 100 and 300 μ*M* AT. The effect of delayed addition of PRL or RU486 on DEX-induced DNA fragmentation is shown in Figure 2. While PRL added at the time of Dex addition and incubated for the entire 4-hr period (i.e., 4-hr PRL incubation) completely blocked DNA fragmentation, delay of PRL for 1 hr (i.e., 3-hr PRL incubation) and 2 hr (i.e., 2-hr incubation) produced a partial inhibition of DNA fragmentation that was related to the duration of PRL exposure (Fig. 2A). Similar results were obtained with delayed addition of RU486 (Fig. 2B).

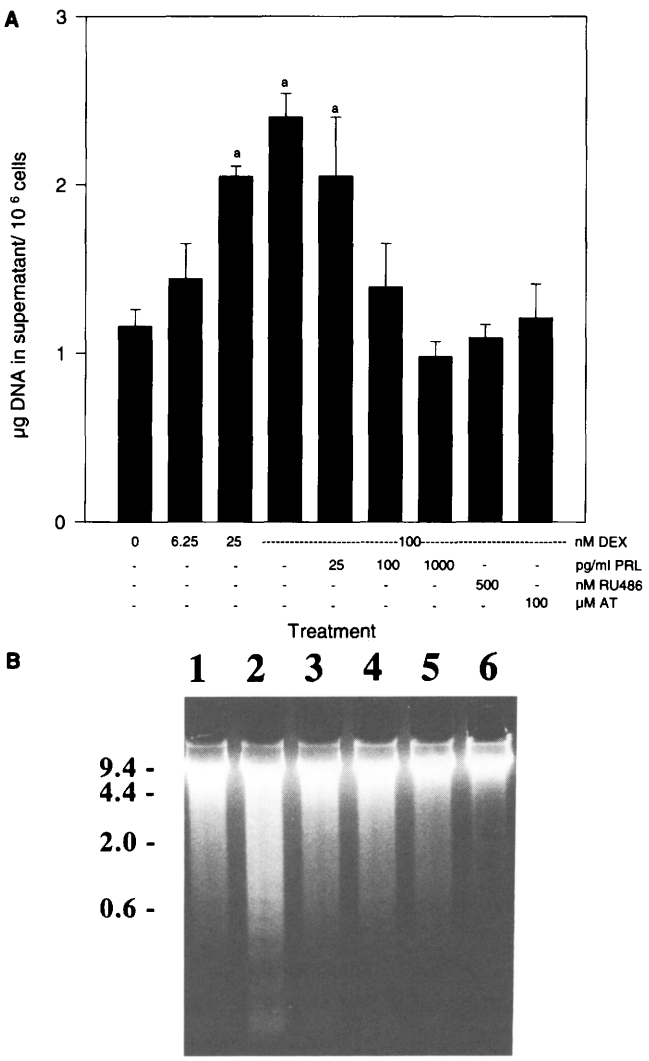

Figure 1. (A) Dexamethasone-induced DNA fragmentation and its inhibition by oPRL, RU486, and AT following a 4-hr incubation of Nb2 cells as measured by reaction with diphenylamine. Data represent mean μg DNA in supernatant ± SE of three to eight individual experiments. [a]Treatment is significantly greater than all other treatments at $P < 0.05$ except those sharing the same symbol. DEX-induced (100 n*M*) DNA fragmentation was significantly blocked by the glucocorticoid receptor antagonist, RU486, the lactogenic hormone, oPRL, and the nuclease inhibitor, aurintricarboxylic acid (AT). (B) An ethidium-bromide stained agarose gel of DNA extracted from Nb2 cells following a 4-hr incubation with DEX and apoptotic inhibitors in serum-free medium. This gel is representative of three individual experiments. (Lane 1) DMSO; (Lane 2 (100 n*M* DEX; (Lane 3) 100 n*M* DEX + 1 ng/ml oPRL; (Lane 4) 100 n*M* DEX + 500 n*M* RU486; (Lane 5) 100 n*M* DEX + 100 μ*M* AT; (Lane 6) 100 n*M* DEX + 300 μ*M* AT. Molecular weight markers expressed in kilobase pairs are indicated to the left of the figure. Dexamethasone shows the "ladder" pattern DNA fragmentation characteristic of apoptosis at 4 hr. Inhibition of DEX-induced DNA fragmentation (banding) is shown by oPRL, RU486, 100 and 300 μ*M* AT.

Protein Kinase C. Two activators of protein ki-

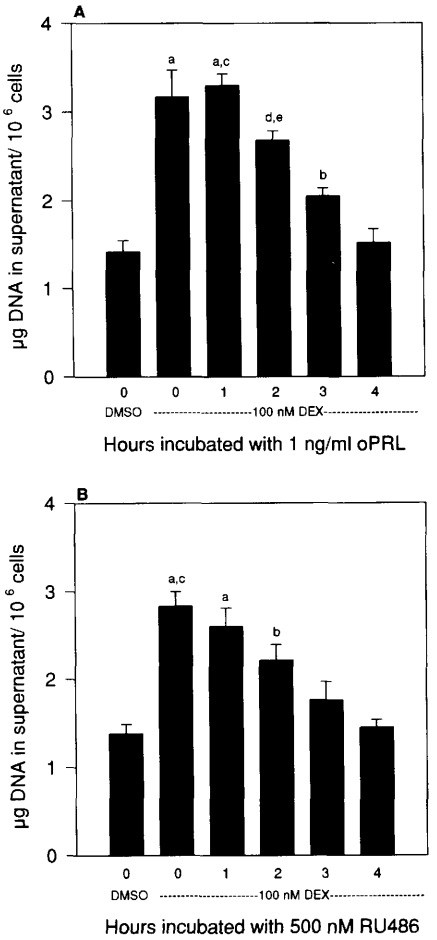

Figure 2. Effect of delayed addition of PRL (1 ng/ml) or RU486 (500 n*M*) on DNA fragmentation after 4-hr exposure to 100 nM DEX. Data represent mean μg DNA in supernatant ± SE of three to four individual experiments. Durations of incubation with oPRL or RU486 of 1, 2, 3, and 4 hr correspond to delays in addition of these agents to the incubation of 3, 2, 1, or 0 hr, respectively. (A) [a]Treatment is significantly different from DMSO, DEX + oPRL for 4 hr, DEX + oPRL for 3 hr at $P < 0.01$; [b]treatment is significantly different from DMSO at $P < 0.05$; [c]treatment is significantly different from DEX + oPRL for 2 hr at $P < 0.05$; [d]treatment is significantly different from DMSO, DEX + oPRL for 4 hr at $P < 0.01$; [e]treatment is significantly different from DEX + oPRL for 1 hr, DEX + oPRL at 3 hr at $P < 0.05$. (B) [a]Treatment is significant different from DMSO, DEX + RU486 at 4 hr, DEX + RU486 at 3 hr at $P < 0.01$; [b]treatment is significantly different from DMSO, DEX + RU486 for 4 hr at $P < 0.01$; [c]treatment is significantly different from DEX + RU486 for 2 hr at $P < 0.05$.

nase C, phorbol-12-myristate-13-acetate (TPA) and 1,2-dioctanoyl-*sn*-glycerol (DiC8), were examined alone or in combination with 200 nM A23187, to determine if they could mimic prolactin inhibition of DEX-induced DNA fragmentation. In preliminary studies, 200 n*M* A23187 was the highest concentration of the ionophore that did not significantly increase basal DNA fragmentation (data not shown). As shown in Figure 3A, the phorbol ester, TPA, alone or in combination with calcium ionophore, A23187, did not inhibit DEX-induced DNA fragmentation. Figure 3B demonstrates that the membrane permeable diacylglycerol, DiC8 alone or in combination with 200 n*M* A23187 does not appear to mimic prolactin.

Two inhibitors of PKC activity, gossypol and quercetin, were examined to determine if they could block prolactin inhibition of DEX-induced DNA fragmentation. As shown in Fig. 3C, gossypol alone significantly increased DNA fragmentation at 25 μ*M*, yet 10 and 25 μ*M* gossypol did not block prolactin inhibition of DEX-induced DNA fragmentation. Quercetin did not block prolactin inhibition of DEX-induced DNA fragmentation, as shown in Figure 3D. Quercetin alone at 50 μ*M* significantly increased basal DNA fragmentation.

Arachidonic Acid Metabolism. To investigate arachidonic acid liberation and eicosanoid production as mediator(s) of prolactin protection against DEX-induced DNA fragmentation, arachidonic acid (AA) was used as a pathway agonist. Figure 4A shows that arachidonic acid exhibited no significant inhibition of DEX-induced DNA fragmentation and thus did not mimic prolactin action. Three inhibitors of arachidonic acid metabolism were examined to determine whether they could block prolactin inhibition of DEX-induced DNA fragmentation. Figure 4B shows that 5,8,11,14-eicosatetraynoic acid (ETYA), a common inhibitor of cyclooxygenases and lipoxygenases, had no effect on prolactin inhibition of DEX-induced DNA fragmentation. Nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor which blocks leukotriene production, increased basal DNA fragmentation at 10–40 μ*M* as shown in Figure 4C. The presence of 1 ng/ml oPRL blocked the combination of DEX and NDGA-induced DNA fragmentation. Indomethacin, a cyclooxygenase inhibitor which blocks subsequent prostaglandin and thromboxane production, did not block prolactin protection as shown in Figure 4D.

Ornithine Decarboxylase and Polyamines. Ornithine decarboxylase (ODC) pathway agonists were tested to determine if they could mimic prolactin protection against DEX-induced DNA fragmentation. As shown in Figure 5A, spermine, the final product of the polyamine pathway, significantly inhibited DEX-induced DNA fragmentation at concentrations of 1.5–2.5 m*M* spermine. However, it is noteworthy that in nine individual incubations with 2.5 m*M* spermine, six totally inhibited, two partially inhibited, and one failed to inhibit DEX-induced DNA fragmentation. Two inhibitors of polyamine production, the ornithine decarboxylase inhibitor, α-difluoromethyl ornithine (DFMO), and the SAM decarboxylase inhibitor, methylglyoxal *bis*(guanylhydrazone) (MGBG) were tested to determine if they could block prolactin inhibition of DEX-induced DNA fragmentation. Figure 5B demonstrates that DFMO did not block prolactin action. MGBG which inhibits the conversion of putrescine to spermidine and spermidine to spermine,

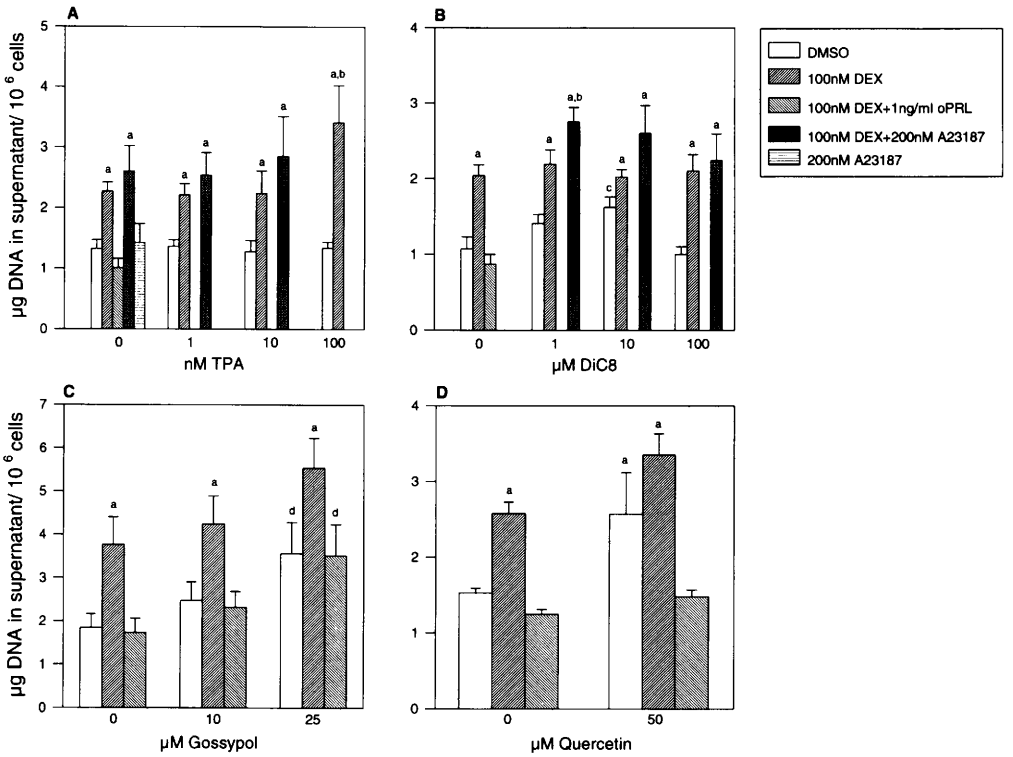

Figure 3. The effect of protein kinase C activators and inhibitors on DEX-induced DNA fragmentation. (A) The effect of phorbol ester, phorbol-12-myristate-13-acetate (TPA), with or without 200 n*M* A23187 on DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of 3–12 individual experiments. [a]Treatment is significantly greater than DMSO and 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$; [b]treatment is significantly greater than 100 n*M* DEX alone at $P < 0.05$. TPA did not mimic prolactin inhibition of DEX-induced DNA fragmentation. (B) The effect of 1,2-dioctanoyl-*sn*-glycerol (DiC8) and 200 n*M* A23187 on DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of four to eight individual experiments. [a,b] Same as in Figure 2A; [c]treatment is significantly greater than 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$. DiC8 did not mimic prolactin inhibition of DEX-induced DNA fragmentation. (C) The effect of gossypol on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of five to seven individual experiments. [a]Same as in Figure 2A; [d]treatments are significantly different than 100 n*M* DEX + 25 μ*M* gossypol but not each other. Gossypol at 25 μ*M* significantly increased basal DNA fragmentation compared to DMSO only. Gossypol did not block prolactin inhibition of DEX-induced fragmentation. (D) The effect of quercetin on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of three to four individual experiments. [a]Treatment is significantly greater than all other treatments except those with the same symbol at $P < 0.05$. Quercetin did not block prolactin inhibition of DEX-induced DNA fragmentation.

shown in Figure 5C, also did not block prolactin inhibition of DEX-induced DNA fragmentation.

Tyrosine Phosphorylation. To evaluate tyrosine kinase activation as a mediator of this prolactin effect, two inhibitors of tyrosine kinases, genistein and tyrphostin-47, were studied. Genistein (25–100 μ*M*), shown in Figure 6A, caused a significant increase in basal DNA fragmentation that was concentration dependent and this effect on DNA fragmentation was significantly attenuated by prolactin. The DNA fragmentation induced at 100 μ*M* genistein in the presence of oPRL was still significantly elevated above that of DMSO and DEX + oPRL controls. Although the DEX response varied when combined with 25–100 μ*M* genistein, this tyrosine kinase inhibitor did not block prolactin inhibition of DEX-induced DNA fragmentation at any concentration. Tyrphostin-47 alone at 100 μ*M* increased basal DNA fragmentation yet tyrphostin-47 did not block prolactin inhibition of DEX-induced DNA fragmentation (Fig. 6B).

Alternatively, the accumulation of phosphotyrosine proteins as the mediator of this prolactin effect was assessed by tyrosine phosphatase inhibition. Inhibitors of tyrosine phosphatase activity, hydrogen peroxide (H_2O_2), orthovanadate, or the more potent combination, pervanadate were tested to determine if phosphotyrosine accumulation mimicked prolactin action. The concentrations of vanadate and H_2O_2 used had no effect on basal DNA fragmentation in 4-hr incubations (data not shown). As shown in Figure 7A, hydrogen peroxide (20 μ*M*) or vanadate (20 μ*M*) alone had no significant effect on DEX-induced DNA fragmentation. In this batch of cells, Nb2 U17 (resurrected 6-24-93), 10 μ*M* H_2O_2 + 10 μ*M* vanadate showed partial inhibition of DEX-induced DNA fragmentation that was significantly greater than DMSO and DEX + oPRL and significantly lower than 100 n*M* DEX. Pervanadate complexes of 20 μ*M* H_2O_2 + 20 μ*M* vanadate essentially blocked DEX-induced DNA fragmentation. Later studies using Nb2 U17 cells (resurrected

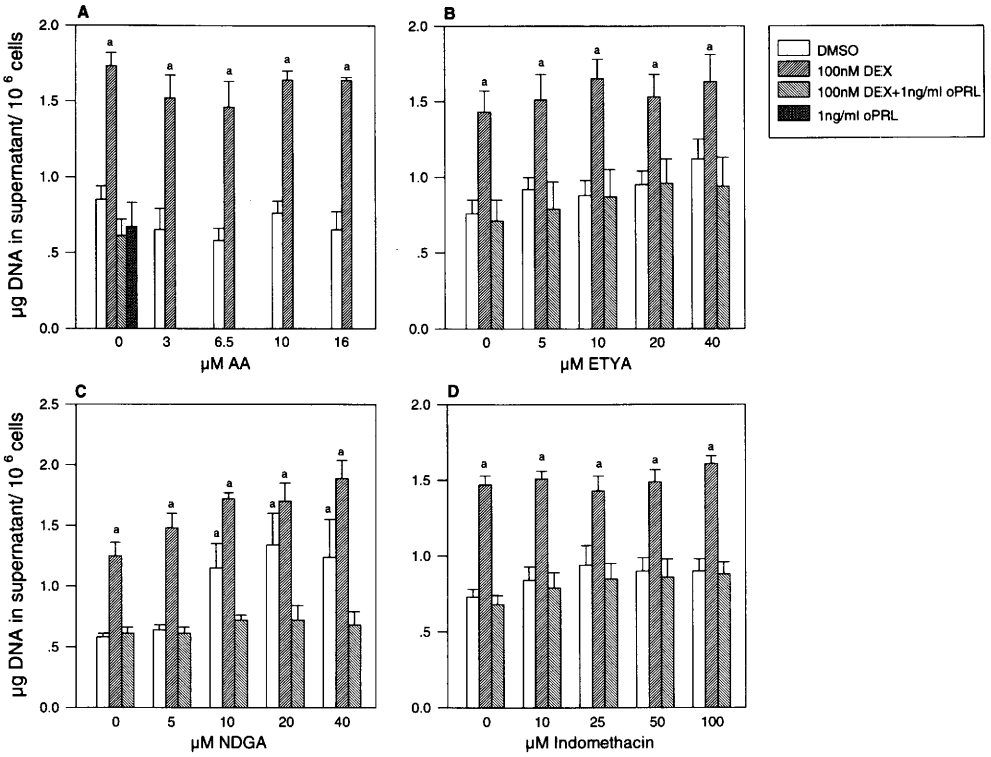

Figure 4. Effects of arachidonic acid and inhibitors of its metabolism on DEX-induced DNA fragmentation. (A) The effect of arachidonic acid on DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of three individual experiments. [a]Treatment is significantly greater than DMSO and 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$. Arachidonic acid did not mimic prolactin inhibit DEX-induced DNA fragmentation. (B) The effect of 5,8,11,14-eisosatetraynoic (ETYA) on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of three to six individual experiments. [a]Same as in Figure 3A. ETYA did not block prolactin inhibition of DEX-induced DNA fragmentation. (C) The effect of nordihydroguaiaretic acid (NDGA) on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of three to six individual experiments. [a]Same as in Figure 3A. NDGA did not block prolactin inhibition of DEX-induced DNA fragmentation. (D) The effect of indomethacin on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean μg DNA in supernatant ± SE of three to six individual experiments. [a]Same as in Figure 3A. Indomethacin did not block prolactin inhibition of DEX-induced DNA fragmentation.

10-19-93) cells exhibited greater sensitivity to H_2O_2 with the optimum pervanadate concentration being 10 μ*M* H_2O_2 + 20 μ*M* vanadate (shown below).

To confirm that pervanadate inhibited DEX-induced apoptosis, the genomic DNA from Nb2 U17 (10-19-93) cells treated with DEX and/or pervanadate for 3 hr was analyzed by agarose gel electrophoresis in Figure 7B. The "ladder" pattern of DNA fragmentation characteristic of apoptosis is induced by 100 n*M* DEX alone. The DNA fragmentation of 100 n*M* DEX was blocked by an optimal concentration of pervanadate as well as with prolactin.

Extracellular Calcium. To investigate the role of extracellular calcium (EC) in prolactin action, it was necessary to determine if DEX action was dependent on EC calcium. Figure 8, A and B, shows that DEX-induced DNA fragmentation occurs in calcium-free PBS (no $CaCl_2$ + 0.1 m*M* EGTA). In calcium-free PBS, prolactin significantly inhibited basal DNA fragmentation. Known blockers of glucocorticoid-induced apoptosis, RU486 and AT, as well as prolactin inhibit DEX-induced DNA fragmentation in calcium-free PBS. Prolactin, RU486, and AT behaved as expected in calcium-supplemented PBS as well (data not shown).

Discussion

The acute assay used in this study in which Nb2 cells are synchronized in G_0/G_1 was modelled after DNA fragmentation assays previously described for quantitation of glucocorticoid-induced cleavage of DNA in thymocytes (8, 30, 40, 41). Although DNA fragmentation was routinely estimated by diphenylamine reactive material released from the genome, the "ladder" pattern of DEX-induced DNA fragmentation was demonstrated by agarose gel electrophoresis confirming an apoptotic process (9). Inhibition of DEX-induced DNA fragmentation by prolactin, the glucocorticoid receptor antagonist, RU486, and nonspecific nuclease inhibitor, aurintricarboxylic acid was consistent with previous reports (7, 40). DEX-induced DNA fragmentation was significantly inhibited at concentrations of 100 pg/ml and 1 ng/ml oPRL which correspond to submaximal and near maximal mitogenic concen-

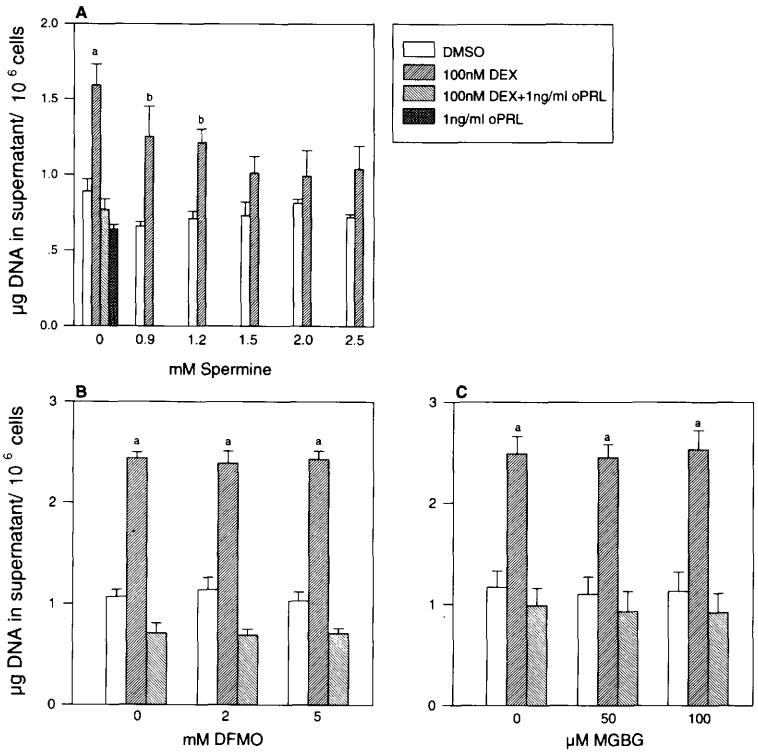

Figure 5. Effects of ornithine decarboxylase pathway product spermine and inhibitors on DEX-induced DNA fragmentation. (A) The effect of spermine on DEX-induced DNA fragmentation. Data represent mean µg DNA in supernatant ± SE of 3–10 individual experiments. [a]Treatment is significantly greater than all other treatments except 100 n*M* DEX + 0.9 m*M* spermine at $P < 0.05$; [b]treatment is significantly greater than 100 n*M* DEX + 1 ng/ml oPRL. Spermine significantly blocked 100 n*M* DEX-induced DNA fragmentation at 1.5–2.5 n*M*. (B) The effect of α-difluoromethyl ornithine (DFMO) on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean µg DNA in supernatant ± SE of three individual experiments. [a]Treatment is significantly greater than DMSO and 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$. DFMO did not block prolactin inhibition of DEX-induced DNA fragmentation. (C) The effect of methylglyoxal *bis*(guanylhydrazone) (MGBG) on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean µg DNA in supernatant ± SE of three individual experiments. [a]Same as in Figure 4B. MGBG did not block prolactin inhibition of DEX-induced DNA fragmentation.

trations of the protein hormone in this cell line (2). Time course data (i.e., delayed addition of PRL) indicate that under these assay conditions the apoptotic action of Dex and the anti-apoptotic action of prolactin are initiated within the 1st hr of their addition.

This assay has several characteristics that make it suited for the study of intracellular signalling associated with the anti-apoptotic action of PRL in Nb2 cells. The use of a chemically defined serum-free medium eliminates the potential influences on apoptosis of unidentified agents in serum. The synchronization of cells in G_0/G_1 reduces the effects of cell cycle differences within the cellular population and accelerates the apoptotic action of glucocorticoid from 12 to 4 hr (7), in agreement with studies of thymocytes *in vitro* and *in vivo* (8, 30, 42). This acceleration of DNA fragmentation suggests that the arrest of cells in G_0/G_1 is permissive for the cytolytic action of glucocorticoid hormones as reported previously (43). The short incubation time minimizes the possibility of biphasic effects of drugs that may occur in longer exposures. Finally, the assay is terminated prior to S phase when the amount of total DNA would increase in cells stimulated by mitogens (1). After the validation of glucocorticoid and prolactin effects in this acute assay system, agonists and antagonists of signaling pathways were examined to gain insight into the intracellular signals mediating the anti-apoptotic effect of PRL.

Protein kinase C (PKC) inhibitors, including gossypol and quercetin (12, 15), blocked prolactin-induced mitogenesis in the Nb2 cell, yet stimulation of PKC by phorbol esters alone was insufficient to induce mitogenesis (11, 12). Phorbol esters alone or in combination with A23187 stimulated prolactin associated events in Nb2 cells such as Na^+/H^+ exchange (44), ornithine decarboxylase activity, and thymidine incorporation (19). In thymocytes, phorbol esters blocked DNA fragmentation and cell death induced by the glucocorticoid methylprednisolone (30). Both TPA (45) and DiC8 (46) blocked ceramide-induced cell apoptosis in myeloid HL-60 cells. In contrast, phorbol esters enhanced cAMP-induced apoptosis in thymocytes (47).

In the current study, however, the most potent phorbol ester, phorbol myristate acetate (TPA), or the cell permeant, diacylglycerol (DiC8), alone or in combination with ionophore to maximize the activation of different PKC isoforms (48), failed to mimic the anti-apoptotic action of PRL. Furthermore, two PKC in-

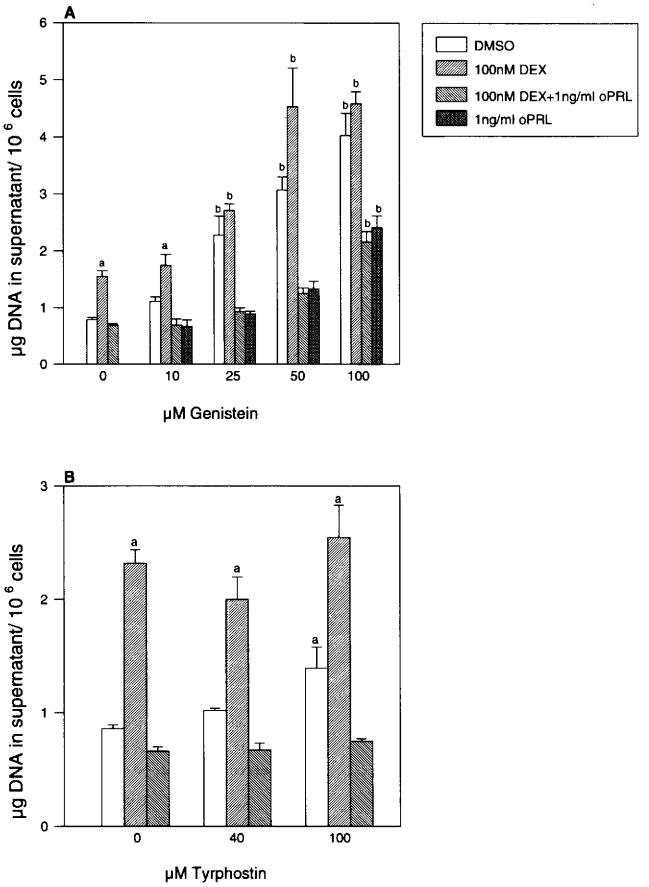

Figure 6. The effect of tyrosine kinase inhibitors on DEX-induced DNA fragmentation. (A) The effect of genistein on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean µg DNA in supernatant ± SE of three to six individual experiments. [a]Treatment is significantly greater than DMSO and 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$; [b]treatment is significantly greater than DMSO, 100 n*M* DEX + 1 ng/ml oPRL, 100 n*M* DEX at $P < 0.05$. Prolactin significantly inhibited DNA fragmentation induced by genistein alone (25–100 µ*M*) and was able to significantly inhibit DNA fragmentation by the combination of DEX and genistein at all concentrations. (B) The effect of tyrphostin-47 on prolactin inhibition of DEX-induced DNA fragmentation. Data represent mean µg DNA in supernatant ± SE of three to four individual experiments. [a]Same as in Figure 5A. Tyrophostin-47 was ineffective in blocking prolactin inhibition of DEX-induced DNA fragmentation.

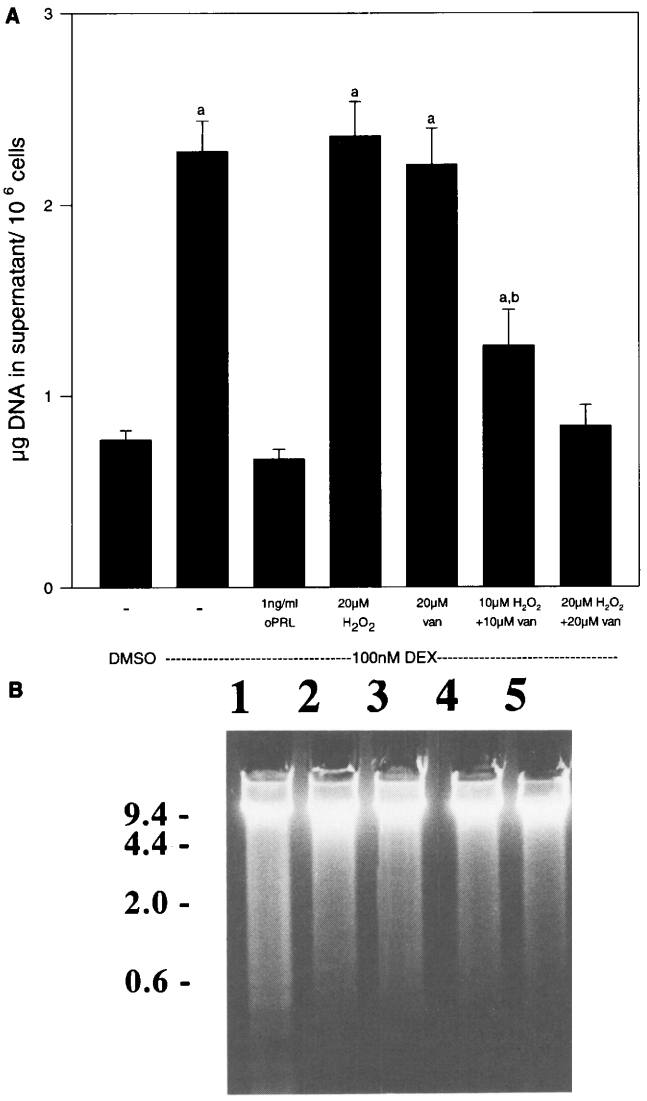

Figure 7. The effects of pervanadate on DEX-induced DNA fragmentation. (A) The effect of hydrogen peroxide, vanadate, and pervanadate on DEX-induced DNA fragmentation in Nb2 U17 cells (resurrected 6-24-93). Data represent mean µg DNA in supernatant ± SE of three to seven individual experiments. [a]Treatment is significantly greater than DMSO and 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$; [b]treatment significantly less than 100 n*M* DEX but significantly greater than DMSO, DEX + oPRL, and DEX + 20 µ*M* H_2O_2/20 µ*M* vanadate (van) at $P < 0.05$. (B) An ethidium-bromide stained agarose gel of DNA extracted from Nb2 U17 cells (resurrected 10-19-93) following incubation with DEX ± pervanadate for 3 hr in serum-free medium. This gel is representative of three individual experiments. (Lane 1) 100 n*M* DEX; (Lane 2) 100 n*M* DEX + 10 µ*M* H_2O_2/20 µ*M* vanadate; (Lane 3) 10 µ*M* H_2O_2/20 µ*M* vanadate; (Lane 4) 100 n*M* DEX + 1 ng/ml oPRL; (Lane 5) DMSO. Molecular weight markers expressed in kilobase pairs are indicated to the left of the figure. In addition to inhibition by prolactin (Lane 4), DEX-induced apoptotic banding (Lane 1) is inhibited by an optimal concentration of pervanadate (Lane 2).

hibitors, gossypol and quercetin, did not block prolactin inhibition of DEX-induced DNA fragmentation. Thus, no compelling evidence exists from these data that PKC mediates the anti-apoptotic action of prolactin. In fact, at 100 n*M*, TPA significantly enhanced DEX-induced DNA fragmentation without affecting basal fragmentation. DiC8 at 10 µ*M*, a concentration that elevated basal DNA supplement, blunted the increment of the DEX effect, but did not prevent DNA fragmentation. The increased DNA fragmentation induced by gossypol and quercetin may reflect depression of basal PKC activity possibly necessary for cell maintenance and tonic suppression of endonuclease activity. Alternatively, it may reflect nonspecific toxic effects of these drugs.

In the Nb2 cell, arachidonic acid (AA) has been shown to be mitogenic and/or enhance prolactin-induced mitogenesis, and prolactin induces prostaglandin formation (13–15). The lipoxygenase inhibitor, NDGA (≥1 µ*M*), decreased prolactin stimulated thy-

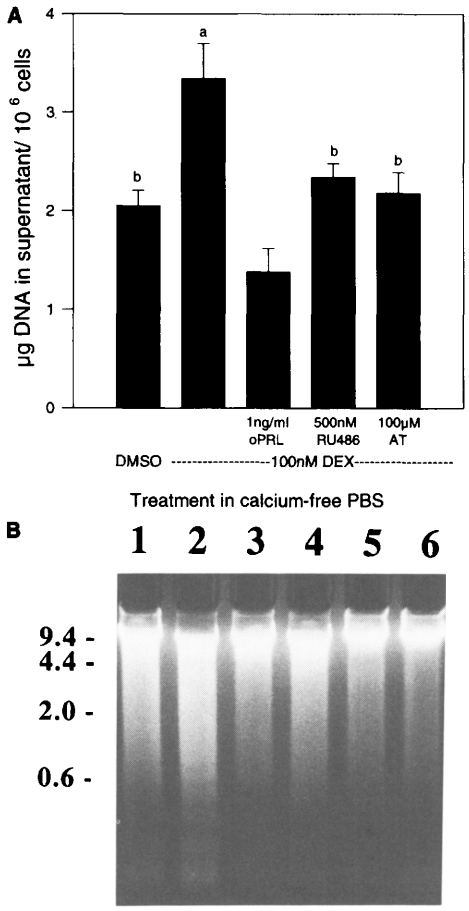

Figure 8. The role of extracellular calcium on prolactin inhibition of DNA fragmentation. (A) The demonstration of DEX-induced DNA fragmentation and its inhibition by oPRL, RU486, and AT in calcium-free PBS. Data represent mean μg DNA in supernatant ± SE of four individual experiments. In calcium-free PBS supplemented with 0.1 m*M* EGTA, basal DNA fragmentation was higher than that typically observed in serum-free medium. In calcium-free PBS, DEX + oPRL was significantly lower than basal DNA fragmentation with DMSO. [a]Treatment is significantly greater than DMSO and 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$; [b]treatment is significantly greater than 100 n*M* DEX + 1 ng/ml oPRL at $P < 0.05$. This demonstrates that DEX-induced DNA fragmentation is not dependent on extracellular calcium. In addition, it also demonstrates that prolactin inhibition of DEX-induced DNA fragmentation is not dependent on extracellular calcium. RU486 and AT inhibited DEX-induced DNA fragmentation but not basal DNA fragmentation. (B) An ethidium-bromide stained agarose gel of DNA extracted from Nb2 cells following a 4-hr incubation with DEX and apoptotic inhibitors in calcium-free PBS supplemented with 0.1 m*M* EGTA. This gel is representative of three individual experiments. (Lane 1) DMSO; (Lane 2) 100 n*M* DEX; (Lane 3) 100 n*M* DEX + 1 ng/ml oPRL; (Lane 4) 100 n*M* DEX + 500 n*M* RU486; (Lane 5) 100 n*M* DEX + 100 μ*M* AT; (Lane 6) 100 n*M* DEX + 300 μ*M* AT. Molecular weight markers expressed in kilobase pairs are indicated to the left of the figure. Dexamethasone shows the "ladder" pattern DNA fragmentation characteristic of apoptosis at 4 hr in calcium-free medium. Inhibition of DEX-induced DNA fragmentation (banding) is shown by oPRL, RU486, 100 and 300 μ*M* AT.

midine incorporation in Nb2 cells (19). Glucocorticoids may exert their inhibitory effects in some immune cells by inhibiting phospholipase A2 liberation of arachidonic acid or prostaglandin synthesizing enzymes (32). Treatment of S49.1 mouse lymphoma cells with NDGA increased their sensitivity to dexamethasone-induced cytolysis (43). Therefore, this pathway was investigated as a possible mediator of the prolactin anti-apoptotic effect using free arachidonic acid and inhibitors of AA metabolism. Free arachidonic acid at concentrations of 3–16 μ*M*, a dose-range previously shown to influence cell mitogenesis (13–15), did not mimic prolactin inhibition of DEX-induced apoptosis nor did inhibitors of arachidonic acid metabolism (49) block prolactin action. Therefore, it appears that eicosanoid production is not essential for the anti-apoptotic effect of prolactin. NDGA significantly increased basal DNA fragmentation by itself, which may be due to inhibition of basal production of lipoxygenase products or a nonspecific cytotoxic effect. This NDGA effect was inhibited by prolactin.

Prolactin stimulates ornithine decarboxylase and SAM decarboxylase activity in the Nb2 lymphoma cell and inhibitors of these enzymes, DFMO and MGBG, respectively, blocked prolactin-induced mitogenesis (21, 22). Glucocorticoid-induced cell death is preceded by decreased polyamine synthesis and growth arrest in mouse lymphoma cells (50). The polyamine, spermine has been shown to be protective against glucocorticoid or A23187-induced DNA fragmentation in thymocytes (31). Our studies reveal that DEX-induced DNA fragmentation was inhibited by spermine at concentrations ranging from 1.5 to 2.5 m*M*, consistent with that protective in thymocytes (31). However, it is unlikely that the ornithine decarboxylase-polyamine pathway mediates the anti-apoptotic action of PRL. Spermidine at nontoxic concentrations (≤0.75 m*M*) was not protective (data not shown). Neither DFMO, which blocks new polyamine production but does not deplete existing spermine, nor MGBG, the SAMDC inhibitor which blocks new spermine production (51), interfered with PRL inhibition of Dex-induced DNA fragmentation. Studies in quiescent Nb2 cells have shown that ODC and SAMDC activities following prolactin addition do not increase until approximately 3 hr following hormone addition (20). Furthermore, lactogen-deprived quiescent Nb2 cells are devoid of detectible ODC activity and produce an inhibitor of ODC which is detectable in the medium for approximately 2 hr after prolactin addition (52). Thus, the production of new spermine is not expected within the first 3 hr following prolactin addition. As discussed above, our time course studies revealed that the prolactin anti-apoptotic action begins within its 1st hr of addition to cells. Positively charged spermine can cause conformational changes in DNA structure (31) which may increase resistance to DNAses (53). Alternatively, PRL may retard the rate of spermine degradation (and, thus, leading to its accumulation) by altering the activity of the rate-limiting enzyme in polyamine degra-

dation, spermidine/spermine N1 acetyltransferase (51). This pathway has yet to be examined in the Nb2 cell.

Several recent studies have shown that prolactin immediately increased tyrosine phosphorylation and tyrosine kinase activity in Nb2 cells (26–28) and that phosphorylation of the tyrosine kinase, JAK2, is one of the earliest events upon prolactin addition (29). Genistein, a flavonoid tyrosine kinase inhibitor blocked prolactin-stimulated tyrosine phosphorylation of a target peptide, as well as prolactin-induced mitogenesis (28). Basal Nb2 cell mitogenesis measured at 72 hr was increased by the tyrosine phosphatase inhibitor ortho-vanadate (10–50 μM) which at 10–25 μM also enhanced mitogenesis due to submaximal concentrations of prolactin (5–10 pg/ml) (27).

We observed that genistein, as well as tyrophostin-47, a nonflavonoid tyrosine kinase inhibitor (54), did not block the ability of prolactin to inhibit DEX-induced DNA fragmentation. In addition, the PKC inhibitor, quercetin, which also inhibits tyrosine kinases at 50 μM (55), did not block prolactin anti-apoptotic action. Although activation of tyrosine kinase does not appear to mediate the anti-apoptotic action of PRL, phosphorylation of tyrosine may participate in the inhibition of DNA fragmentation. We observed that the tyrosine phosphatase inhibitor, pervanadate, was extremely effective in inhibiting Dex-inducing apoptosis. Pervanadate is potentially 100- to 1000-fold more potent than its components, hydrogen peroxide and vanadate (56). In fact, hydrogen peroxide ($\leq$20 μM) and vanadate ($\leq$40 μM) alone had no effect on DNA fragmentation (data not shown). On the other hand, okadaic acid (0.1–10 nM), an inhibitor of serine/threonine phosphatases (phosphatases 1 and 2A) (57) had no influence on Dex-induced DNA fragmentation in the absence or presence of oPRL (25 pg/ml) (data not shown). Both H_2O_2 and vanadate in millimolar concentrations can stabilize the glucocorticoid receptor and reduce the ability of glucocorticoid hormones to transform the receptor to the DNA binding state (58). We have demonstrated that prolactin and pervanadate when preincubated with cells for 1 hr are both effective in inhibiting DNA fragmentation induced by the calcium ionophore, A23187 (250–500 nM) (34). Thus prolactin and pervanadate exhibit their anti-apoptotic effects against a nonglucocorticoid agent as well.

In our experience higher concentrations of H_2O_2 ($\geq$50 μM) were acutely cytolytic to Nb2 cells and pervanadate itself increased basal cell death of cells by 24 hr (data not shown). These cytotoxic effects could reflect the fact that H_2O_2 and vanadate induce DNA strand breaks (59) or that prolonged and unregulated inhibition of tyrosine phosphatase activity is detrimental to cell viability.

Whether PRL exerts its anti-apoptotic action via inhibition of tyrosine dephosphorylation remains to be determined. The fact that PRL inhibited DEX-induced apoptosis in the presence of tyrosine kinase inhibition (i.e., genistein, tyrphostin, and quercetin) is not inconsistent with this possibility. Considerable tyrosine kinase activity has been demonstrated (50% of baseline) in extracts of Nb2 cells at a dose of genistein that blocks PRL-induced tyrosine phosphorylation and mitogenesis (28). Furthermore, we have observed that pervanadate, like PRL, inhibited Dex-induced DNA fragmentation in the presence of genistein (data not shown). In the current study, the increased DNA fragmentation observed at selected doses of quercetin (50 μM), genistein (25–100 μM), and tyrphostin (100 μM) could reflect depression of basal tyrosine kinase activity. Genistein also acts at the ATP binding site of topoisomerase II which has homology to that of tyrosine kinase (60). Thus, apoptosis might be due to depression of topoisomerase II, as has been proposed for the apoptotic effect of genistein in immature thymocytes (61).

Of two genes that are expressed in Nb2 cells within 15 min of PRL addition, c-*myc* and interferon regulatory factor-1 (IRF-1) (62), genistein blocks only the former (L.-Y. Yu-Lee, personal communication). The anti-apoptotic action of PRL may be linked to early IRF-1 expression which peaks at 1 hr in G_1 (63). IRF-1 expression is associated with immunoreactive STAT91 (64), a DNA binding protein activated by tyrosine phosphorylation (65). STAT91 may correspond to the p90/97 protein in Nb2 cells demonstrated to be tyrosine phosphorylated within minutes of prolactin addition (3, 26).

Glucocorticoid-induced apoptosis of lymphoid tissues is believed to be dependent on calcium from either intracellular or extracellular sources (41). In thymocytes, glucocortioid-mediated cytolysis is dependent on extracellular calcium (41), while in lymph node lymphocytes the process can occur in the absence of extracellular calcium (66). The role of extracellular calcium in DEX-induced apoptosis and its inhibition by PRL was examined in Nb2 cells incubated in a calcium-free medium. Under these conditions, DEX was capable of inducing apoptosis, consistent with the situation of lymph node lymphocytes (66). Furthermore, PRL was capable of blocking DEX-induced apoptosis, as well as spontaneous DNA fragmentation, in calcium-free medium. Interestingly, early PRL-stimulated events occur in Nb2 cells in reduced calcium medium even though subsequent mitogenesis was blocked under these conditions (17). The role of intracellular calcium in the hormonal modulation of apoptosis of Nb2 cells remains to be determined.

It is noteworthy that in several instances reported herein, agents previously reported to impair PRL-induced mitogenesis failed to influence the anti-

apoptotic effect of PRL. This is consistent with our previous findings suggesting divergent pathways for the control of mitogenesis and apoptosis (7, 37). Future studies will focus on the role of tyrosine phosphorylation and calcium in the hormonal control of apoptosis of Nb2 cells.

This work was supported in part by research grants from the Gustavus and Louise Pfeiffer Research Foundation (Redlands, CA) and the Grant-in-Aid Program for Faculty of Virginia Commonwealth University to R.J.W.

The authors wish to thank the following individuals: Dr. Joseph Feher of Virginia Commonwealth University for his assistance in measuring calcium in incubation media and for providing TPA; Dr. Mohammed Kalimi of Virginia Commonwealth University for providing RU 486; Dr. Roger Deraedt of Roussel-Uclaf for supplying RU 486 to Dr. Kalimi; Dr. Jitrendra Dave of the Walter Reed Army Institute of Research for providing DFMO; Dr. Joseph P. Liberti of Virginia Commonwealth University for providing Nb2 lymphoma cells; and Dr. Peter W. Gout of the Cancer Control Agency of British Columbia for providing Nb2 lymphoma cells. The authors are also grateful to the National Hormone and Pituitary Program for their gift of ovine prolactin.

1. Noble RL, Gout PW, Beer CT. Prolactin-dependent Nb rat lymphomas and their responses to hormones *in vivo* and *in vitro*. In: Hollander VP, Ed. Hormonally Responsive Tumors. Orlando: Academic Press, p63, 1985.
2. Tanaka T, Shiu RPC, Gout PW, Beer CT, Noble RL, Friesen HG. A new sensitive and specific bioassay for lactogenic hormones: Measurement of prolactin and growth hormone in human serum. J Clin Endocrinol Metab **51**:1058–1063, 1980.
3. Rui H, Djeu JY, Evans GA, Kelly PA, Farrar WL. Prolactin receptor triggering. Evidence for rapid tyrosine kinase activation. J Biol Chem **267**:24076–24081, 1992.
4. Croze F, Walker A, Friesen HG. Stimulation of growth of Nb2 lymphoma cells by interleukin-2 in serum-free and serum-containing media. Mol Cell Endocrinol **55**:253–259, 1988.
5. Sadeghi H, Wang SB. Proliferation of Nb2 lymphoma cells *in vitro* in response to interleukin-7. Immunol Lett **34**:105–108, 1992.
6. Fleming WH, Pettigrew NM, Matusik RJ, Friesen HG. Thymic origin of the prolactin-dependent Nb2 lymphoma cell line. Cancer Res **42**:3138–3142, 1982.
7. Fletcher-Chiappini SE, Compton MM, LaVoie HA, Day EB, Witorsch RJ. Glucocorticoid-prolactin interactions in Nb2 lymphoma cells: Antiproliferative versus anticytolytic effects. Proc Soc Exp Biol Med **202**:345–352, 1993.
8. Wyllie AH. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature **284**:555–556, 1980.
9. Wyllie AH, Morris RG, Smith AL, Dunlop D. Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol **142**:67–77, 1984.
10. Carter-Su C, Argetsinger LS, Campbell GS, Wang X, Ihle J, Witthuhn B. The identification of JAK2 kinase as a signalling molecule for growth hormone. Proc Soc Exp Biol Med **206**:210–215, 1994.
11. Gertler A, Walker A, Friesen HG. Enhancement of human growth hormone stimulated mitogenesis of Nb2 node lymphoma cells by 12-O-tetradecanoyl-phorbol-13-acetate. Endocrinology **116**:1636–1644, 1985.
12. Rillema JA, Waters SB, Tarrant TM. Studies on the possible role of protein kinase C in the prolactin regulation of cell replication in Nb2 node lymphoma cells. Proc Soc Exp Biol Med **192**:140–144, 1989.
13. Ofenstein JP, Rillema JA. Possible involvement of the phospholipases in the mitogenic actions of prolactin (PRL) on Nb2 lymphoma cells. Proc Soc Exp Biol Med **185**:147–152, 1987.
14. Hafez MM, Costlow ME. Phosphatidylethanolamine turnover is an early event in the response of Nb2 lymphoma cells to prolactin. Exp Cell Res **148**:37–43, 1989.
15. Sylvester PW, Ip MM, Briski KP. Effects of specific fatty acids on prolactin-induced Nb2 lymphoma cell proliferation. Life Sci **52**:1977–1984, 1993.
16. Ganguli S, Hu LB, Menke PT, Freeman JJ, Collier RJ, Gertler A. Prolactin induces calcium flux and rapid translocation of protein kinase c (PKC) to the nucleus in Nb2 lymphoma cells. Endocrin Soc Abstr **268**:91, 1990.
17. Murphy PR, Dimattia GE, Friesen HG. Role of calcium in prolactin-stimulated c-*myc* gene expression and mitogenesis in Nb2 lymphoma cells. Endocrinology **122**:2476–2485, 1988.
18. Buckley AR, Montgomery DW, Kibler R, Putnam CW, Zukoski CF, Gout PW, Beer CT, Russell DH. Prolactin stimulation of ornithine decarboxylase and mitogenesis in the Nb2 node lymphoma cells: The role of protein kinase C and calcium mobilization. Immunopharmacology **12**:37–51, 1986.
19. Russell DH, Buckley AR, MOntgomery DW, Larson NA, Gout PW, Beer CT, Putnam CW, Zukoski CF, Kibler R. Prolactin-dependent mitogenesis in the Nb2 node lymphoma cell: Effects of immunosuppressive cyclopeptides. J Immunol **138**:276–284, 1987.
20. Richards JF, Beer CT, Bourgeault C, Chen K, Gout PW. Biochemical response of lymphoma to mitogenic stimulation by prolactin. Mol Cell Endocrinol **26**:41–49, 1982.
21. Elsholtz P, Shiu RPC, Friesen HG. Role of ornithine decarboxylase in proliferation of prolactin-dependent lymphoma cells. Biochem Cell Biol **64**:381–386, 1986.
22. Ofenstein JP, Rillema JA, Lawson DM. Prolactin stimulation of Nb2 node lymphoma cell division is inhibited by polyamine biosyntheis inhibitors. Proc Soc Exp Biol Med **180**:236–239, 1985.
23. Kornberg LJ, Liberti JP. Lactogen-induced protein phosphorylation in Nb2 cells. Arch Biochem Biophys **279**:109–115, 1990.
24. Clevenger CV, Torigoe T, Reed JC. Prolactin induces the rapid phosphorylation and activation of the prolactin receptor associated *raf*-1 kinase in a T-cell line. Endocrin Soc Abstr **1626**:457, 1993.
25. Shechter Y, Sakal E, Cohen R, Cohen-Chapnik N, Gertler A. Hydroxyphenyl acetate derivatives inhibit protein tyrosine kinase activity and proliferation in Nb2 rat lymphoma cells and insulin-induced lipogenesis in rat adipocytes. Mol Cell Endocrinol **80**:183–192, 1991.
26. Rillema JA, Campbell GS, Lawson DM, Carter-Su C. Evidence for a rapid stimulation of tyrosine kinase activity by prolactin in Nb2 rat lymphoma cells. Endocrinology **131**:973–975, 1992.
27. Fan G, Carbajo P, Rillema JA. Possible role of tyrosine kinases in the prolactin stimulation of cell division in Nb2 node lymphoma cells. Horm Metab Res **25**:256–258, 1993.
28. Carey GB, Liberti JP. Inhibition of tyrosine kinase activity abolishes anabolic and mitogenic effects of prolactin and growth hormone in Nb2 cells. Biochem Mol Biol Int **31**:635–642, 1993.
29. Rui H, Kirken RA, Farrar WL. Activation of receptor-associated tyrosine kinase JAK2 by prolactin. J Biol Chem **269**:5364–5368, 1994.
30. McConkey DJ, Hartzell P, Jondal M, Orrenius S. Inhibition of DNA fragmentation in thymocytes and isolated nuclei by agents that stimulate protein kinase C. J Biol Chem **264**:13399–13402, 1989.
31. Brune B, Hartzell P, Nicotera P, Orrenius S. Spermine prevents

endonculease activation and apoptosis in thymocytes. Exp Cell Res **195**:323–329, 1991.
32. Bailey JM. New mechanisms for effects of anti-inflammatory glucocorticoids. Biofactors **3**:97–102, 1991.
33. LaVoie HA, Witorsch RJ. Investigations of intracellular signals mediating the anti-cytolytic action of prolactin in Nb2 lymphoma cells. Endocrin Soc Abst **1232**:358, 1993.
34. LaVoie HA, Witorsch RJ. Pervanadate inhibits dexamethasone-induced apoptosis in prolactin-dependent Nb2 lymphoma cells. Endocrin Soc Abstr **1767**:642, 1994.
35. Horsman DE, Masui S, Gout PW. Karyotypic changes associated with loss of prolactin dependency of rat Nb2 note lymphoma cell cultures. Cancer Res **51**:282–287, 1991.
36. Walker A, Croze F, Friesen HG. A serum-free medium for culturing lactogen dependent and autonomous NB2 node lymphoma cells. Endocrinology **120**:2389–2397, 1987.
37. Witorsch RJ, Day EB, LaVoie HA, Hashemi N, Taylor JK. Comparison of glucocorticoid-induced effects in prolactin-dependent and autonomous rat Nb2 lymphoma cells. Proc Soc Exp Biol Med **203**:454–460, 1993.
38. Burton K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J **62**:315–323, 1956.
39. Dulbecco R, Vogt M. Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp Med **99**:167–182, 1954.
40. McConkey DJ, Hartzell P, Nicotera P, Orrenius S. Calcium-activated DNA fragmentation kills immature thymocytes. FASEB J **3**:1843–1849, 1989.
41. McConkey DJ, Nicotera P, Hartzell P, Bellomo G, Wyllie AH, Orrenius S. Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. Arch Biochem Biophys **269**:365–370, 1989.
42. Compton MM, Cidlowski JA. Rapid *in vivo* effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. Endocrinology **118**:38–45, 1986.
43. Bittner S, Wielckens K. Glucocorticoid-induced lymphoma cell growth inhibition: The role of leukotriene B_4. Endocrinology **123**:991–1000, 1988.
44. Too CKL, Walker A, Murphy PR, Cragoe EJ Jr., Jacobs HK, Friesen HG. Identification of amiloride-sensitive Na^+/H^+ exchange in rat NB2 lymphoma node lymphoma cells. Stimulation by 12-O-tetradecanoyl-phorbol-13-acetate. Endocrinology **121**:1503–1511, 1987.
45. Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. Science **259**:1769–1771, 1993.
46. Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA, Grant S. Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc Natl Acad Sci USA **91**:73–77, 1994.
47. Suzuki K, Kizaki H, Tadakuma T, Ishimura Y. 12-O-tetradecanoylphorbol 13-acetate potentiates the action of cAMP in inducing DNA cleavage in thymocytes. Biochem Biophys Res Commun **171**:827–831, 1990.
48. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science **258**:607–614, 1992.
49. Parker CW, Stenson WF, Huber MG, Kelly JP. Formation of thromboxane B2 and hydroxyarachidonic acids in purified human lymphocytes in the presence and absence of PHA. J Immunol **122**:1572–1577, 1979.
50. Russell DH, Haddox MK, Gehring U. Effects of dexamethasone and dibutyryl cyclic AMP on polyamine synthesizing enzymes in mouse lymphoma cells. J Cell Physiol **106**:375–384, 1981.
51. Pegg AE. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J **234**:249–262, 1986.
52. Richards JF, Bishop PB, Peng T, Beer CT, Gout PW. An inhibitor of ornithine decarboxylase in lactogen-deprived Nb2 node rat lymphoma cells. Biochim Biophys Acta **883**:542–551, 1986.
53. Snyder RD. Polyamine depletion is associated with altered chromatin structure in HeLa cells. Biochem J **260**:697–704, 1989.
54. Gillespie J, Dye JF, Schachter M, Guillou PJ. Inhibition of pancreatic cancer cell growth *in vitro* by the tyrphostin group of tyrosine kinase inhibitors. Br J Cancer **68**:1122–1126, 1993.
55. Shisheva A, Shechter Y. Quercetin selectively inhibits insulin receptor function *in vitro* and the bioresponses of insulin and insulinomimetic agents in rat adipocytes. Biochemistry **31**:8059–8063, 1992.
56. Fantus IG, Kadota S, Deragon G, Foster B, Posner BI. Pervanadate (peroxide[s] of vanadate) mimics insulin action in rat adipocytes via activation of the insulin receptor tyrosine kinase. Biochemistry **28**:8864–8871, 1989.
57. Mumby MC, Walter G. Protein serine/threonine phosphatases: Structure, regulation, and functions in cell growth. Physiol Rev **73**:673–699, 1993.
58. Pratt WB. Transformation of glucocorticoid and progesterone receptors to the DNA binding state. J Cell Biochem **35**:51–68, 1987.
59. Birnboim HC. A superoxide anion induced DNA strand-break metabolic pathway in human leukocytes: Effects of vanadate. Biochem Cell Biol **66**:374–381, 1988.
60. Markovits J, Linassier C, Fosse P, Couprie J, Pierre J, Jacquemin-Sablon A, Saucier J-M, LePecq J-B, Larsen AK. Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Res **49**:5111–5117, 1989.
61. McCabe MJ Jr., Orrenius S. Genistein induces apoptosis in immature thymocytes by inhibiting topoisomerase-II. Biochem Biophys Res Commun **194**:944–950, 1993.
62. Yu-Lee L-y, Stevens AM, Hrachovy JA, Schwartz LA. Prolactin-mediated regulation of gene transcription in lymphocytes. Ann NY Acad Sci **594**:146–155, 1990.
63. Schwarz LA, Stevens AM, Hrachovy JA, Yu-Lee L-y. Interferon regulatory factor-1 is inducible by prolactin, interleukin-2, and concanavalin a in T cells. Mol Cell Endocrinol **86**:103–110, 1992.
64. Yu-Lee L-y, Sieger KA, Stevens AM, Wang Y-f, Roan F, Axtell SA, O'Neal KD. Prolactin signaling molecules. Endocrin Soc Abstr **532**:333, 1994.
65. Shual K, Ziemiecki A, Wilks AF, Harpur AG, Sadowski HB, Gilman MZ, Darnell JE. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature **366**:580–583, 1993.
66. Kaiser N, Edelman IS. Further studies on the role of calcium in glucocorticoid-induced lymphocytolysis. Endocrinology **103**:936–942, 1978.