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Induction of Apoptotic Cell Death in Hen Granulosa Cells by Ceramide*

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ABSTRACT

Recent studies have demonstrated that ovarian follicle atresia occurs extensively before follicle selection into the avian preovulatory hierarchy, and that this process is mediated via granulosa cell apoptosis. Subsequent to follicle selection, granulosa cells are inherently resistant to apoptosis, and such resistance is correlated with increased expression of death suppressor genes such as *bcl*-xlong. In the present studies we used this avian ovary model system to 1) identify cellular characteristics and mechanisms related to apoptotic cell death of granulosa cells *in vitro*, and 2) further characterize functional differences between apoptosis-susceptible (4- to 8-mm follicle) and apoptosis-resistant (preovulatory follicle) granulosa cells. Treatment of granulosa cells from the largest preovulatory follicle with *N*-octanoylsphingosine (C8-ceramide) results in pronounced oligonucleosome formation, a hallmark of apoptosis. That this is indicative of programmed cell death is supported by an increased incidence of pyknotic nuclei and apoptotic bodies in C8-ceramide-treated samples compared to that in control cultured cells. Tumor necrosis factor-α, a stimulator of ceramide production, actively promotes oligonucleosome formation in apoptosis-susceptible, but not in apoptosis-resistant, granulosa cells. Induction of apoptosis is also observed after exposure of apoptosis-resistant granulosa cells to sphingomyelinase treatment and UV irradiation, which are known to stimulate endogenous ceramide production, and to the anticancer drug, daunorubicin, which initiates *de novo* ceramide biosynthesis via activation of ceramide synthase. Although treatment of granulosa cells with fumonisin B1, a specific ceramide synthase inhibitor, blocks daunorubicin-stimulated oligonucleosome formation, UV-induced cell death is unaffected. Taken together, these results demonstrate that pharmacological factors known to mimic the actions of ceramide or stimulate ceramide production can induce oligonucleosome formation and programmed cell death in granulosa cells. More importantly, however, the ability of a physiologically relevant initiator of ceramide biosynthesis, tumor necrosis factor-α, to promote cell death is evident only in apoptosis-susceptible granulosa cells collected from atresia-prone prehierarchal follicles. These data provide support for ceramide as an important intracellular signaling mechanism, mediating granulosa cell apoptosis and follicle atresia. (*Endocrinology* **137:** 5269–5277, 1996)

A MAJORITY of primordial and early developing ovarian follicles in both avian and mammalian species is eliminated throughout the female's reproductive life via follicle atresia (1), and recent studies have demonstrated that apoptotic cell death is the molecular mechanism underlying this process (1–3). Oligonucleosome formation, as identified and quantitated by DNA 3′-end labeling methods, is a key event associated with apoptosis (4). Internucleosomal DNA cleavage together with morphological criteria (*e.g.* formation of pyknotic nuclei and apoptotic bodies, and cell shrinkage) can, therefore, be used as markers to elucidate the regulatory events associated with the onset of apoptotic cell death.

Virtually all cells undergoing apoptosis during follicular atresia in the hen ovary are of granulosa cell origin (3, 5). Recent work from our laboratory has identified two populations of granulosa cells that are differentially sensitive to apoptosis induced by tropic hormone deprivation: granulosa cells from follicles that inherently exhibit a high incidence of atresia (prehierarchal follicles $<$9 mm in diameter) are highly susceptible to apoptotic cell death, whereas granulosa cells from follicles that under normal physiological conditions do not undergo atresia (preovulatory follicles) are comparatively resistant to apoptosis (3). Extracellular factors such as FSH, vasoactive intestinal peptide, and transforming growth factor-α attenuate the progression of apoptotic cell death in apoptosis-sensitive granulosa cells (3, 6). Moreover, granulosa cell resistance to apoptosis is correlated with increased expression of the death suppressor gene, *bcl*-xlong compared to that in apoptosis-sensitive cells (3). Although the mechanisms by which extracellular survival factors and death-suppressing genes support granulosa cell survival have been the object of much investigation, comparatively less attention has been focused on factors that render prehierarchal follicle granulosa cells susceptible to cell death. Two potential mechanisms of action, which may not be mutually exclusive, include activation of interleukin-converting enzyme-related proteases and the initiation of ceramide biosynthesis (7, 8).

Ceramide has recently emerged as an important mediator of the effects of extracellular agents on cell growth, differentiation, and programmed cell death in a growing number of cell types (9–11). Apoptotic cell death activated by tumor necrosis factor-α (TNFα), Fas (APO-1/CD95), and UV and ionizing radiation is mediated by the sphingomyelin cycle (12–14). This evolutionarily conserved signaling pathway links specific cell surface receptors and environmental stressors to the nucleus, presumably via the stress-activated protein kinase pathway (14–16). Ceramide biosynthesis is initiated by hydrolysis of the phospholipid, sphingomyelin,

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which is preferentially localized to the outer leaflet of the plasma membrane. Sphingomyelin hydrolysis occurs within seconds to minutes via the action of sphingomyelinase, a membrane-localized enzyme (17, 18). Alternatively, stimulation of ceramide synthase, an enzyme that facilitates *de novo* ceramide synthesis, increases intracellular ceramide levels and mediates apoptosis in cells treated with the anthracycline, daunorubicin (19). Such ceramide production can be blocked by a naturally occurring specific inhibitor of ceramide synthase, fumonisin B1 (19). Ceramide generated by either sphingomyelin breakdown or synthase activation subsequently serves as a second messenger and under appropriate circumstances can lead to oligonucleosome formation and apoptotic cell death (20). The specificity of ceramide as a second messenger in this activity has been demonstrated by the finding that cell-permeable ceramide analogs (*e.g.* N-octanoylsphingosine), but not analogs of related lipid second messengers (*i.e.* 1,2-diacylglycerol and arachadonic acid), are able to mimic the cell death-inducing effects of TNFα, Fas, and ionizing radiation (13).

Details of the apoptosis-inducing pathway downstream of ceramide generation have yet to be elucidated, although several immediate targets for ceramide action have been identified. Matthias *et al.* (21) characterized a 97-kDa proline-directed serine/threonine kinase, termed ceramide-activated protein kinase, that is activated after interaction of ligand with TNFα and interleukin-1β receptors and is closely coupled to receptor activation (22). A ceramide-activated serine/threonine protein phosphatase has also been identified and may signal through activation of protein kinase Cζ (23). Downstream factors, including Raf (24), mitogen-activated protein kinase (25), and activating protein-1 binding proteins, as well as down-regulation of c-*myc* (26), stimulation of the stress-activated protein kinase pathway (14–16), phosphorylation and inactivation of I-κB (23, 27), and nuclear translocation of nuclear factor-κB (28–30) have all been implicated in mediating the actions of ceramide in various cell systems.

Therefore, the present experiments were conducted to further define the characteristics associated with apoptosis-sensitive *vs.* -resistant granulosa cells and to address the cellular mechanisms by which granulosa cells are induced to undergo cell death via apoptosis. In light of mounting data implicating the sphingomyelin pathway and ceramide production as functional components of apoptotic cell death in nonreproductive tissues, the effect of this lipid second messenger on inducing cell death was evaluated in granulosa cells from atresia-susceptible or -resistant hen ovarian follicles. The results of these studies further define a novel model system in which to study molecular aspects of cellular resistance to apoptotic cell death and should eventually contribute to increased understanding of ovarian tumor development.

Materials and Methods

Animals and reagents

Single comb White Leghorn hens (H&H Poultry, Portland, IN), 25–40 weeks of age and laying in regular sequences of at least five or six eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layeena Mash, Purina Mills, St. Louis, MO) and water, and exposed to a photoperiod of 15 h of light, 9 h of darkness, with lights on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h before a midsequence ovulation by cervical dislocation. All procedures described below were reviewed and approved by the University of Notre Dame institutional animal care and use committee and were performed in accordance with the principles explained in the Guide for the Care and Use of Laboratory Animals.

N-Octanoylsphingosine (C8-ceramide), *N*-acetylsphingosine (C2-ceramide), and the inactive ceramide analog, C2-dihydroceramide (*N*-acetylsphinganine) were obtained from Biomol (Plymouth Meeting, PA), prepared as 5-mM stock solutions in 100% ethanol, and stored at −20 C until use. Recombinant murine (rm) TNFα was obtained from R&D (Minneapolis, MN), prepared as a 10 μg/ml stock solution in sterile saline, and stored at −20 C until use. Additional reagents were obtained from Sigma Chemical Co., unless otherwise stated.

Granulosa cell cultures

The first experiment was conducted to establish the plating efficiencies of apoptosis-sensitive *vs.* -resistant granulosa cells in primary culture. Pure populations of granulosa cells from the largest preovulatory (F1) follicle and 20–25 prehierarchal (4–8 mm) follicles were harvested from multiple hens and dispersed in 0.3% type 2 collagenase (Worthington Corp., Freehold, NJ) in medium 199 (M199)-HEPES for 30 min at 37 C. Additional mechanical dispersion with a sterile pipette was necessary to achieve a single cell suspension. Cells were plated overnight at a density of 1.25×10^6/ml in M199-HEPES supplemented with 2.5% FBS (Life Technologies, Grand Island, NY), 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite in 100-mm petri dishes (Corning, Corning, NY). The following day, cultures were washed once with fresh medium, and adherent cells were removed via trypsinization. After centrifugation at 200 × *g* for 5 min and resuspension in M199-HEPES, viable cells were quantitated in triplicate samples by the trypan blue exclusion method. Plating efficiencies (percentages) were calculated by dividing the number of viable cells after 24 h of culture by the total number of viable cells seeded on the previous day. Results were obtained from replicate cell preparations (n = 4) for each granulosa cell type, collected from 3 different hens.

For studies evaluating the effect of ceramide on apoptosis-resistant granulosa, cells from the F1 follicle were isolated and plated overnight in serum-supplemented M199-HEPES medium. The following morning, adherent cells were washed once and cultured with 5 or 50 μM C8-ceramide, 50 μM C2-ceramide, or 50 μM C2-dihydroceramide for up to 24 h. Medium was subsequently removed from each plate, and adherent cells were collected by trypsinization and pelleted by centrifugation. Samples of plated untreated cells were trypsinized, washed with M199-HEPES and immediately frozen (t24 control). All cell pellets were stored at −70 C before DNA isolation.

Granulosa cell incubations

Granulosa cells were collected from 20–25 prehierarchal (4- to 8-mm) follicles, as previously described (31). Due to the rapid progression of apoptotic cell death and, thus, the low plating efficiency (as determined in the first experiment) all experiments with granulosa cells from 4- to 8-mm follicles were conducted under suspension incubation conditions. Aliquots of cells were immediately frozen at −70 C (t0) or incubated in 12 × 75-mm polypropylene culture tubes in the presence of 167 nM phorbol 12-myristate 13-acetate (PMA), 1–5 mM 8-bromo-cAMP (8-br-cAMP), 40 μM C8-ceramide, 10 or 100 ng/ml rmTNFα, or combinations of these in serum-free medium (M199-HEPES; Life Technologies) (31) for 6 h. After incubations, cells were pelleted by gentle centrifugation (200 × *g*, 5 min, room temperature) and frozen at −70 C until genomic DNA was extracted.

To further characterize the induction of cell death in apoptosis-resistant granulosa cells under suspension incubation conditions, cells from the F1 follicle were isolated and collagenase-dispersed as described above, and subsequently incubated in suspension culture with sphingomyelinase (from *Bacillus cereus*; 100 mU/ml), 8-br-cAMP (1 mM), PMA (167 nM), C8-ceramide (40 μM), combinations of 40 μM C8-ceramide and 8-br-cAMP or PMA, rmTNFα (10 or 100 ng/ml), daunorubicin (200 nM), and fumonisin B1 (100 μM) in serum-free M199-HEPES. Cells were

incubated at 37 C for up to 24 h, pelleted, and then frozen at −70 C before genomic DNA isolation.

UV treatment of F1 granulosa cell cultures

To assess the effects of UV radiation on granulosa cells in culture, cells from the F1 follicle were harvested, dispersed, and plated overnight in 100-mm petri dishes at a density of 1.25×10^6 cells/ml (4 ml/dish) in serum-supplemented M199-HEPES, as described above. The following morning, medium was removed from the cells, and adherent cells were irradiated with 120,000 μJ/cm^2 UV light using a Stratalinker UV cross-linker (model 1800, Stratagene, La Jolla, CA). Medium was immediately replaced, and cells were further cultured at 37 C for 3–24 h.

To analyze the potential protective effects of PMA (167 nM), 8-br-cAMP (1 mM), or fumonisin B1 (100 μM) on UV-induced cell death, cells were pretreated for 1 h at 37 C in the absence or presence of each agent. Medium was removed during irradiation (as described above) and then replaced (with the same treatment) for an additional 24 h. After culture, medium was again removed from each plate, and adherent cells were collected by trypsinization and pelleted by centrifugation. All cell pellets were stored at −70 C before DNA isolation.

Preparation of genomic DNA and evaluation of oligonucleosome formation

Genomic DNA was prepared from all treated samples and corresponding controls as previously reported (3, 32). After isolation and quantitation of DNA, 1 μg/sample was 3′-end labeled with [α-^{32}P]dideoxy (dd)-ATP (3000 Ci/mmol; Amersham, Arlington Heights, IL) using the terminal transferase reaction, and the degree of low mol wt (LMW; <20 kilobases) DNA was analyzed by autoradiography, as previously described (5, 32).

Morphological evaluation of apoptosis

To confirm and evaluate the incidence of induced apoptosis *in vitro*, granulosa cells from F1 follicles were collected by trypsinization after a 6-h treatment in culture with 50 μM C8-ceramide, 50 μM C2-dihydroceramide, or ethanol vehicle alone. Cells were fixed in Dietrich's fixative overnight, dehydrated, and embedded in paraffin. Sections (5 μm) were placed onto SuperFrost Plus slides (Fisher Scientific, Fairlawn, NJ) and stained with hematoxylin/picric acid methyl blue, as previously described (3). Apoptotic cells were identified by the presence of pyknotic nuclei and apoptotic bodies, quantitated by determining the average number of apoptotic cells (of 100 total cells) in five random areas of triplicate samples, and photographed.

Data analysis

All treatments of granulosa cells from both F1 and 4- to 8-mm follicles were performed at least in triplicate, using different hens. The degree of LMW (<20 kilobases) DNA labeling in multiple trials was analyzed by autoradiography and β-scintillation counting as previously described (3, 5, 32). Levels of oligonucleosome formation (fold increase *vs.* the appropriate control) are presented as the mean ± SEM of quantitated data from replicate experiments. Data were analyzed using a paired *t* test or one-way ANOVA, with significant interactions ($P < 0.05$) partitioned using Fisher's protected least significant difference multiple range test (Statview, Abacus Concepts, Berkeley, CA).

Results

Plating efficiency of 4- to 8-mm vs. F1 follicle granulosa cells

Granulosa cells from the F1 follicle exhibit a significantly greater plating efficiency in M199-HEPES supplemented with 2.5% FBS than granulosa cells from prehierarchal (4–8 mm) follicles (80 ± 5% plating efficiency *vs.* 30 ± 1%, respectively; $P < 0.05$). With both stages of follicle development, granulosa cells that successfully plate form viable primary cultures.

C8-ceramide-induced apoptosis in F1 follicle granulosa cells

Gel electrophoresis of ^{32}P 3′-end-labeled DNA revealed that exposure of primary culture F1 granulosa cells to 50 μM C8-ceramide induced DNA fragmentation with a pattern indicative of internucleosomal cleavage (Fig. 1A). The effects of C8-ceramide treatment were first detected after 6 h of culture and increased with longer treatment (1.4 ± 0.3-fold increase *vs.* t0 levels at t3; 6.4 ± 1.4-fold at t6, $P < 0.05$ *vs.* unincubated cells; and 14 ± 1.8 and 20.8 ± 4.2-fold increases by t12 and t24, respectively, $P < 0.001$ *vs.* t0; Fig. 1B). Such oligonucleosome formation was not observed in the presence of 5 μM C8-ceramide, 50 μM C2-ceramide, 50 μM C2-dihydroceramide (the inactive ceramide analog), or ethanol vehicle alone (fold increase ± SEM for LMW DNA labeling *vs.* freshly collected cells (5 μM C8, 1.3 ± 0.3; 50 μM C2, 2.8 ± 0.5; 50 μM dihydro-C2, 1.3 ± 0.2; $P > 0.05$ *vs.* t0).

The appearance of morphological features characteristic of apoptotic cell death was monitored in F1 follicle granulosa cells treated with ceramide analogs or carrier alone. Neither the presence of C2-dihydroceramide (50 μM) nor ethanol vehicle alone had any significant effect on F1 follicle granulosa cell morphology after 6 h of culture compared to that

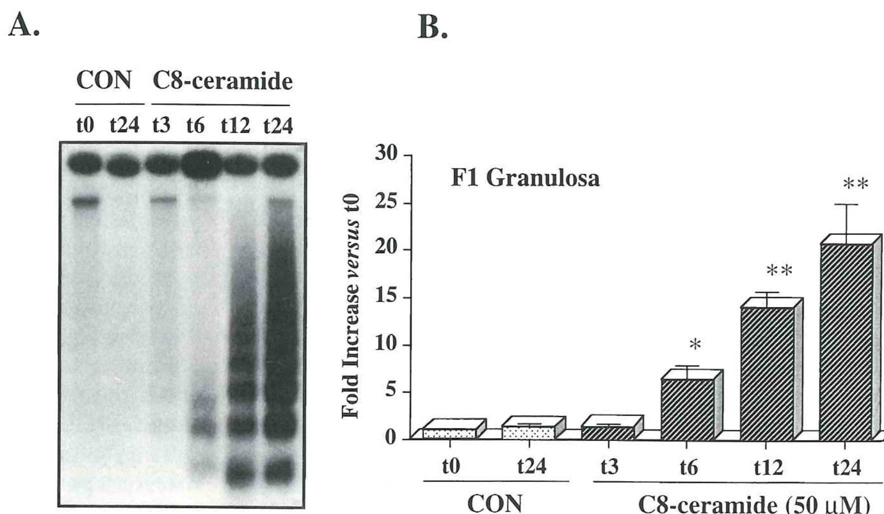
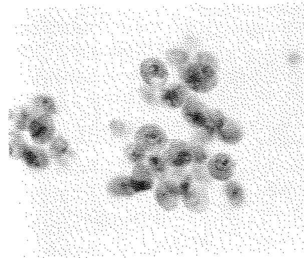
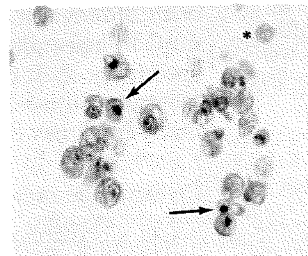


FIG. 1. C8-ceramide-induced cell death in F1 follicle granulosa cells. A, Representative autoradiograph of oligonucleosome formation in F1 granulosa cells after treatment with 50 μM C8-ceramide for 3–24 h (t3 to t24) in culture compared to those in freshly collected cells (t0 CON) and untreated cultured cells (t24 CON). Genomic DNA was extracted, radiolabeled with [^{32}P]ddATP, resolved by gel electrophoresis, and then analyzed by autoradiography. B, Data represent the fold increase ± SEM (n = 3 replicate experiments) in labeled LMW DNA compared to that in t0 cells determined by β-scintillation counting of labeled LMW DNA (<20 kilobases). *, $P < 0.05$; **, $P < 0.001$ (*vs.* t0).

A. CONTROL



B. C8-CERAMIDE



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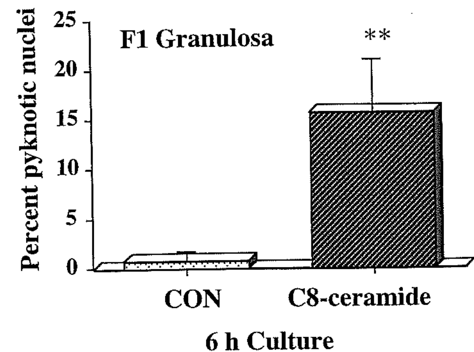


FIG. 2. Incidence of apoptotic cell death in primary culture F1 follicle granulosa cells cultured for 6 h in the absence (A; control) or presence (B) of C8-ceramide. Cells were stained with hematoxylin/picric acid methyl blue as described in *Materials and Methods*. *Arrows* indicate pyknotic nuclei. *, Apoptotic bodies. Magnification, ×400. C, The percentage of apoptotic cells in control and C8-ceramide-treated cultures. Values represent the mean ± SEM for apoptotic cells (of 100 total cells) in 5 random areas from triplicate samples. **, $P < 0.001$.

in samples of freshly collected F1 follicle granulosa cells (data not shown). By contrast, cells treated with 50 μM C8-ceramide for an identical length of time demonstrated an increased incidence of apoptotic cell death [17.9 ± 6.0% *vs.* 0.85 ± 1.2% in cultured (t6) controls; $P < 0.001$] characterized by the presence of pyknotic nuclei and apoptotic bodies (Fig. 2).

Oligonucleosome formation after sphingomyelinase treatment of F1 follicle granulosa cells

To support the contention that F1 follicle granulosa cells are susceptible to apoptotic cell death after the induction of endogenous ceramide production, the effects of this lipid-mediated signaling pathway were evaluated by incubating cells with sphingomyelinase for 3–24 h. Gel electrophoresis of ^{32}P 3′-end-labeled DNA revealed oligonucleosome formation in primary F1 follicle granulosa cells after 24 h of exposure to sphingomyelinase (100 mIU/ml; Fig. 3; 22.8 ± 4.5-fold increase at t24 *vs.* freshly collected cells; $P < 0.05$).

Regulation of granulosa cell apoptosis by pharmacological factors

In subsequent studies, the ability of several pharmacological agents to attenuate oligonucleosome formation in hen granulosa cells was evaluated. Figure 4 illustrates the effects of a cAMP analog (8-br-cAMP), a protein kinase C activator (PMA), and combinations of these reagents with C8-ceramide on cell death. No biochemical indications characteristic of apoptosis were observed after treatment of F1 follicle granulosa cells with 8-br-cAMP [2.0 ± 0.4-fold increase *vs.* unincubated (t0) cells; $P > 0.05$; Fig. 4A]. By contrast, C8-ceramide induced oligonucleosome formation in F1 follicle granulosa cells (4.9 ± 1.2-fold increase after 6 h *vs.* t0; $P < 0.05$), and 8-br-cAMP failed to reverse the death-inducing effects of C8-ceramide ($P > 0.05$ *vs.* C8-ceramide alone).

In accord with previously reported data (3), 8-br-cAMP attenuated the progression of apoptosis in granulosa cells

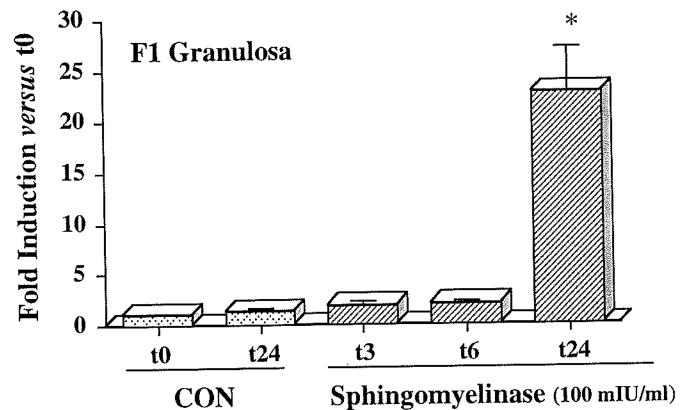


FIG. 3. Induction of oligonucleosome formation by sphingomyelinase in F1 follicle granulosa cells. Cells were freshly collected (t0 CON) or isolated and incubated in M199-HEPES in the absence (t24 CON) or presence of 100 mU/ml sphingomyelinase over a period of 24 h (t3 to t24). Genomic DNA was extracted, radiolabeled with [^{32}P]ddATP, resolved by gel electrophoresis, and then analyzed by autoradiography. Data represent the fold increase ± SEM (n = 3 replicate experiments) in labeled LMW DNA compared to that in t0 cells. *, $P < 0.05$; *vs.* t0.

from 4- to 8-mm follicles (13.2 ± 1.8-fold *vs.* 18.6 ± 1.8-fold increase in t6 control; $P < 0.05$; Fig. 4B). C8-ceramide (40 μM) potentiated oligonucleosome formation in granulosa cells from prehierarchal follicles (34.8 ± 8.7-fold increase) compared to the t6 control ($P < 0.05$) and reversed the attenuation of apoptotic cell death (34.9 ± 7.8-fold increase) observed with 8-br-cAMP treatment alone (Fig. 4B).

Finally, to investigate the potential protective effects of enhanced protein kinase C activity on spontaneous and ceramide-induced cell death in the hen ovary, granulosa cells from 4- to 8-mm and F1 follicles were treated with PMA (167 nM) for a period of 6 h. This protein kinase C activator failed to attenuate the pronounced spontaneous apoptosis ob-

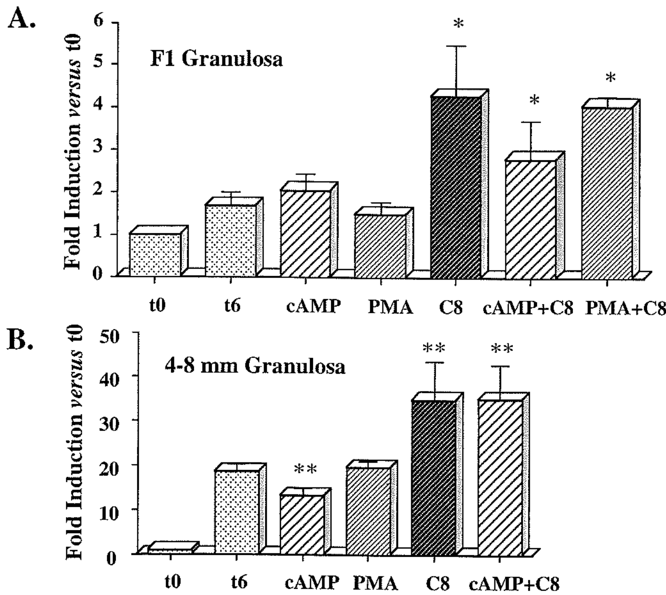


FIG. 4. Effects of 8-br-cAMP (cAMP) and PMA on granulosa cells undergoing spontaneous or C8-ceramide-induced oligonucleosome formation. Granulosa cells from F1 and 4- to 8-mm follicles were isolated and incubated in the absence (t6) or presence of treatments in M199-HEPES at 37 C for 6 h. Genomic DNA was extracted and labeled with [^{32}P]ddATP as described in Fig. 1. A, F1 follicle granulosa cells incubated with 8-bromo-cAMP (cAMP; 1 mM) or PMA (167 nM), C8-ceramide (C8; 40 μM), or combinations of C8 and cAMP or PMA. Cells were pretreated with cAMP (1 mM) or PMA (167 nM) for 1 h before the addition of C8. B, Granulosa cells from 4- to 8-mm follicles were pretreated with cAMP (1 mM) or PMA (167 nM) for 1 h before the addition of C8. Results are presented as the fold increase ± SEM (n = 3 replicate experiments) in labeled LMW DNA compared to that in freshly collected (t0) cells. *, $P < 0.05$ *vs.* t0; **, $P < 0.05$ *vs.* t6.

served in 4- to 8-mm follicle granulosa cells after 6 h in culture. Furthermore, PMA did not alter the level of oligonucleosome formation compared to the t6 control treatment and failed to inhibit C8-ceramide-induced oligonucleosome formation in F1 follicle granulosa cells (Fig. 4, A and B).

Influence of TNFα on granulosa cell apoptosis

To assess the effects of TNFα on granulosa cell apoptosis, cells from 4- to 8-mm and F1 follicles were collected and incubated in the presence of rmTNFα for a period of 6 or 24 h, respectively (Fig. 5). Neither 10 nor 100 ng/ml rmTNFα induced oligonucleosome formation in F1 follicle granulosa cells after a 24-h treatment [1.0 ± 0.2-fold and 1.1 ± 0.2-fold increase *vs.* freshly collected (t0) cells for 10 and 100 ng/ml rmTNFα, respectively]; as a positive control, additional cells treated concomitantly with C8-ceramide (50 μM) demonstrated a 20.8 ± 4.2-fold increase in oligonucleosome formation compared to t0 controls (Fig. 5A). By comparison, rmTNFα (100 ng/ml) potentiated oligonucleosome formation in 4- to 8-mm follicle granulosa cells compared to the level of spontaneous oligonucleosome formation observed after 6 h in M199-HEPES (30.2 ± 3.5-fold increase in the presence of 100 ng/ml rmTNFα *vs.* 20.7 ± 5.9-fold increase in t6 control samples; $P < 0.05$; Fig. 4B). Moreover, rmTNFα-treatment of 4- to 8-mm follicle granulosa cells reversed the protective effects of 8-br-cAMP to enhance oligonucleosome

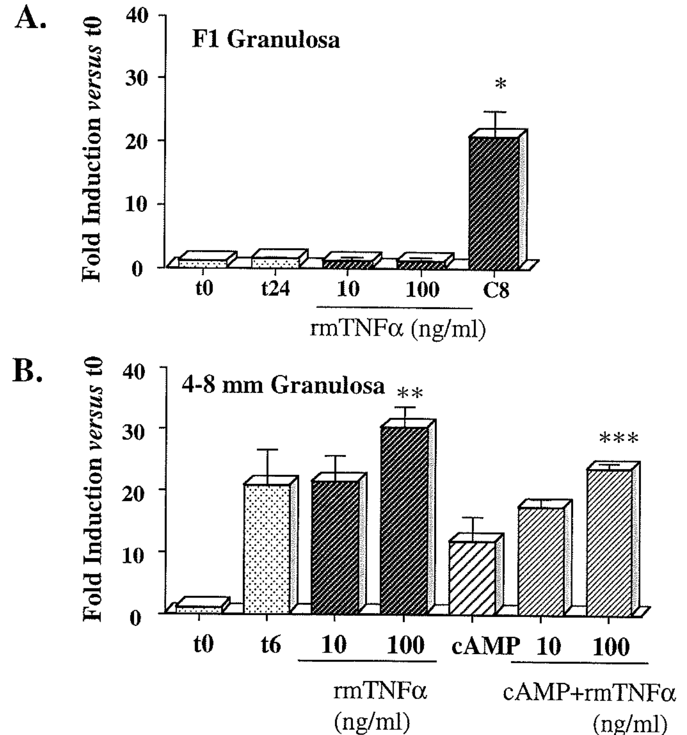


FIG. 5. The effects of TNFα on oligonucleosome formation in granulosa cells are dependent upon the stage of follicle differentiation. Granulosa cells from F1 and 4- to 8-mm follicles were isolated and incubated for 24 or 6 h, respectively. Genomic DNA was subsequently extracted and labeled with [^{32}P]ddATP as described in Fig. 1. A, F1 granulosa cells incubated in the absence (t24) or presence of rmTNFα (10 or 100 ng/ml) or C8-ceramide (50 μM). B, Granulosa cells from 4- to 8-mm follicles were incubated in the absence (t6) or presence of rmTNFα (10 or 100 ng/ml) or were pretreated with 8-br-cAMP (cAMP; 1 mM) for 1 h before the addition of rmTNFα. Data represent the fold increase ± SEM (n = 3 replicate experiments) in labeled LMW DNA compared to that in unincubated (t0) cells. *, $P < 0.05$ *vs.* unincubated t0; **, $P < 0.05$ *vs.* t6; ***, $P < 0.05$ *vs.* cAMP.

formation (23.7 ± 0.9-fold increase in the presence of 8-br-cAMP plus 100 ng/ml rmTNFα *vs.* 11.8 ± 4.0-fold with 8-br-cAMP alone; $P < 0.05$).

UV-induced apoptosis in F1 granulosa cells

A single dose of UV irradiation (120,000 μJ/cm^2) induced oligonucleosome formation in primary cultures of F1 follicle granulosa cells after 24 h of culture (17.4 ± 3.5-fold induction *vs.* freshly collected cells; $P < 0.05$; Fig. 6). On the other hand, pretreatment of cultured cells with 8-br-cAMP, but not PMA, for 1 h before UV irradiation completely prevented oligonucleosome formation in UV-treated cells.

Effects of fumonisin on daunorubicin- and UV-induced oligonucleosome formation

To assess potential biosynthetic pathways by which ceramide production and cell death may be stimulated in primary cultures of granulosa cells, apoptosis-resistant cells from the F1 follicle were incubated for 24 h in the absence or presence of daunorubicin (200 nM), fumonisin B1 (100 μM), or a combination of these two pharmacological agents. Significant oligonucleosome formation was observed in the

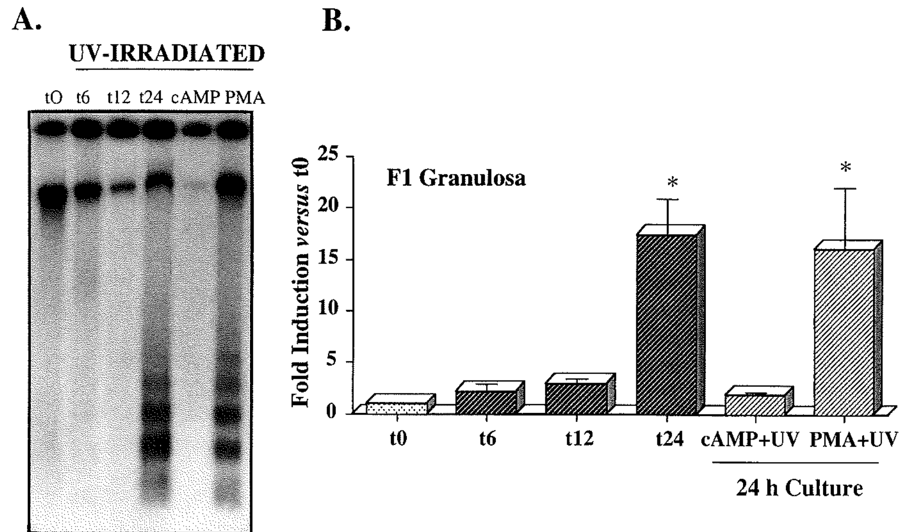
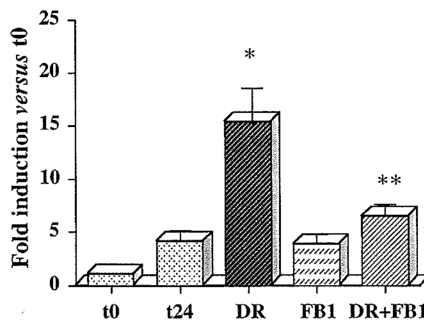


FIG. 6. Time course of UV-induced apoptosis in F1 follicle granulosa cells. A, Oligonucleosome formation in F1 granulosa cells over 24 h in culture after a single dose of UV irradiation (120,000 μJ/cm^2; t0, plated, UV-treated, then cells immediately collected; t6, t12, and t24, cells collected 6, 12, or 24 h, respectively, after UV radiation). F1 granulosa cells were also irradiated after a 1-h pretreatment with either 8-br-cAMP (cAMP; 1 mM) or PMA (167 nM); media plus appropriate treatments were replaced for a 24-h incubation at 37 C. Genomic DNA was subsequently isolated and labeled with [^{32}P]ddATP as described in Fig. 1. B, Fold increase ± SEM in labeled LMW DNA compared to that in unincubated (t0) cells (n = 3 replicate experiments). *, $P < 0.05$ *vs.* t0.



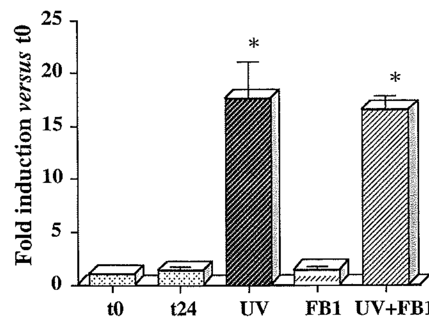


FIG. 7. Differential effects of fumonisin B1 on daunorubicin- and UV-induced oligonucleosome formation in F1 follicle granulosa cells treated for 24 h. A, Cells were incubated in the absence (t24) and presence of daunorubicin (DR; 200 nM), fumonisin B1 (FB1; 100 μM), or a combination of the two (where cells were pretreated for 1 h with FB1 and then cotreated with DR and FB1 for 24 h). B, F1 granulosa cells were plated overnight and subjected to UV irradiation as described in Fig. 6. Cells were collected immediately after UV irradiation (t24) or 24 h after UV irradiation (UV) or fumonisin B1 (FB1) treatment, or were preincubated with 100 μM FB1 for 1 h before UV irradiation, and then medium containing FB1 was replaced for a 24-h incubation at 37 C. Data represent the fold increase ± SEM (n = 3 replicate experiments) in labeled LMW DNA compared to that in unincubated (t0) controls. *, $P < 0.05$ *vs.* t24; **, $P < 0.05$ *vs.* DR.

presence of daunorubicin alone (15.4 ± 3.2-fold increase; Fig. 7A). A significant inhibition of oligonuclesome formation was observed in daunorubicin-treated cells preincubated for 1 h and subsequently cotreated with fumonisin B1 for 24 h (6.5 ± 1.2-fold increase; $P < 0.05$ *vs.* daunorubicin alone). By contrast, no attenuation of oligonucleosome formation was observed in UV-irradiated F1 follicle granulosa cells treated similarly with fumonisin B1 (Fig. 7B).

Discussion

The predominant cell type that undergoes apoptosis during follicular atresia in the hen is the epithelial-derived granulosa cell (5). Recent work from our laboratory has demonstrated that granulosa cells isolated from various stages of follicular development exhibit differing characteristics with regard to susceptibility or resistance to apoptotic cell death. In the present studies, granulosa cells from 4- to 8-mm follicles demonstrated a limited ability to form a primary culture when plated in medium with a minimal serum component compared to granulosa cells from the F1 follicle. In light of our recent data showing that incubation of 4- to 8-mm follicles in defined medium for 6 h results in extensive apoptotic cell death specifically within the granulosa layer (3), it is concluded that the limited plating efficiency is due to inherent susceptibility to apoptosis. Furthermore, we recently demonstrated that viable, 4- to 8-mm follicle granulosa cells that successfully adhere after 24 h of culture express significantly higher levels of *bcl*-xlong messenger RNA compared to freshly collected cells (33). Thus, it is tempting to speculate that granulosa cells from 4- to 8-mm follicles forming a primary culture are inherently resistant to apoptosis and may have originated from prehierarchal follicles that were destined for follicle selection, whereas those undergoing apoptosis may have originated from preatretic follicles.

The results from our studies also document the ability of the sphingomyelin/ceramide signaling pathway to induce apoptosis *in vitro* in otherwise resistant F1 follicle granulosa cells and to accelerate or potentiate apoptosis in 4- to 8-mm

follicle granulosa cells that are inherently susceptible to programmed cell death. Both biochemical (oligonucleosome formation) and morphological (formation of pyknotic nuclei and apoptotic bodies) indications of apoptotic cell death are evident in F1 follicle granulosa cells within 6 h after the addition of 50 μM C8-ceramide. Similarly, treatment with sphingomyelinase and UV irradiation, both of which have previously been demonstrated to enhance endogenous production of ceramide in a variety of cell types (11–14, 16, 17, 34–37), markedly induces oligonucleosome formation after 24 h of treatment. By contrast, treatment of the cells with an equimolar concentration of a related ceramide analog, C2-ceramide, or 5 μM C8-ceramide fails to induce oligonucleosome formation, whereas the inactive ceramide analog, C2-dihydroceramide, induces neither oligonucleosome formation nor morphological indications of apoptosis. Such results indicate cell-specific effects of C8-ceramide within avian granulosa cells. It is of interest to note, however, that there is an apparent difference in the time to onset of oligonucleosome formation in C8-ceramide-treated *vs.* sphingomyelinase- and UV-treated F1 follicle granulosa cells. It is suggested that the longer time to induction is due either to the intensity of the signal generated (a pharmacological agonist *vs.* endogenously produced ceramide) or to different kinetics of sphingomyelinase- and UV-generated ceramide formation.

Previous studies with isolated granulosa cells or whole follicles from gonadotropin-primed prepubertal rats have demonstrated an attenuation of apoptosis when incubated in the presence of gonadotropins (FSH and LH/hCG) or growth factors (*i.e.* transforming growth factor-α, insulin-like growth factor I, and basic fibroblast growth factor) (3, 6, 38–41). The effects of these autocrine/paracrine/endocrine factors are thought to be mediated at least in part by the adenyl cyclase-cAMP and tyrosine kinase intracellular signaling systems. Similarly, recent work has demonstrated that granulosa cells isolated from hen prehierarchal (4- to 8-mm) follicles spontaneously undergo programmed cell death within 6 h under conditions of tropic hormone deprivation, whereas oligonucleosome formation is attenuated by the presence of the cAMP analog, 8-br-cAMP (3). Although in the present studies, pretreatment with 8-br-cAMP was capable of rescuing F1 follicle granulosa cells from UV-induced cell death, equimolar concentrations of 8-br-cAMP failed to attenuate the apoptosis-inducing effects of C8-ceramide in F1 or in 4- to 8-mm follicle granulosa cells. These results may again be explained by the difference between the pharmacological actions of micromolar concentrations of the exogenously added ceramide analog, C8-ceramide, compared to picomolar concentrations of endogenously generated ceramide (19).

The inability of physiological factors such as gonadotropins or growth factors to completely suppress oligonucleosome formation in either chicken granulosa cells or rat ovarian follicles indicates that multiple mechanisms functioning in parallel are required to maintain follicle viability. An alternative protective pathway may be mediated by protein kinase C, the activation of which has been shown to be correlated with cell proliferation and survival in several cell types (42). Conversely, studies in primary rat hepatocytes have demonstrated that a decrease in protein kinase C activity is associated with an increase in programmed cell death (43). Activation of protein kinase C in hen granulosa cells has previously been shown to inhibit steroidogenesis and induce plasminogen activator activity (44). In the present study, however, PMA treatment failed to either attenuate the spontaneous progression of apoptosis in 4- to 8-mm follicle granulosa cells or rescue F1 follicle granulosa cells from C8- or UV-induced cell death. These results indicate that protein kinase C activation alone is not sufficient to influence cell survival or death-inducing pathways in granulosa cells from either prehierarchal or preovulatory follicles.

TNFα has been demonstrated to induce rapid sphingomyelin hydrolysis to ceramide by the action of neutral sphingomyelinase in several cell lines, including granulosa cells (45–47). This cytokine, produced in the mammalian ovary by macrophages, white blood cells, oocytes, and follicular cells, has been shown to mediate such processes as follicular development, atresia, and ovulation (48–50). An additional role for TNFα in the mediation of granulosa cell death is suggested by the present observations that rmTNFα enhances both spontaneous and 8-br-cAMP-attenuated oligonucleosome formation in 4- to 8-mm follicle granulosa cells within 6 h of treatment. It is also of significance to note that unlike the effects of UV irradiation in F1 follicle granulosa cells, TNFα promotes oligonucleosome formation in prehierarchal follicle granulosa cells in a time frame similar to that of C8-ceramide.

By comparison, rmTNFα does not induce oligonucleosome formation in F1 follicle granulosa cells after a 24-h culture period. The differential effects of TNFα that are related to stage of follicle differentiation may be due to a greater expression of the TNFα R-1 receptor in 4- to 8-mm *vs.* F1 follicle granulosa cells. On the other hand, it is possible that the constitutively elevated levels of *bcl*-xlong messenger RNA in F1 follicle granulosa cells (compared to 4- to 8-mm follicle granulosa cells) (3) provide resistance to TNFα-induced cell death, as overexpression of the related death-suppressing gene, *bcl*-2, has recently been reported to block ceramide-induced cell death in a leukemia cell line (8). Studies are currently underway to discriminate between these two possibilities.

Evidence from multiple cell types demonstrates that apoptotic cell death as a result of UV and ionizing radiation involves sphingomyelin hydrolysis and elevation of endogenous ceramide levels (12–14). Alternatively, ceramide can be generated within a cell by activation of the biosynthetic enzyme, ceramide synthase. For instance, daunorubicin-induced apoptosis in a human leukemia cell line is mediated via *de novo* ceramide synthesis and can be almost completely blocked by the presence of the specific ceramide synthase inhibitor, fumonisin B1 (19). In the present studies, the ability of fumonisin B1 to markedly attenuate daunorubicin-induced, but not UV-mediated, oligonucleosome formation in F1 follicle granulosa cells suggests that *de novo* biosynthesis of ceramide is not a requirement for UV-induced apoptosis.

In summary, activation of the ceramide signaling pathway by treatment with C8-ceramide, sphingomyelinase, daunorubicin, or UV irradiation induces apoptotic cell death in granulosa cells. Our results also demonstrate that the ability

of a physiological ligand (TNFα) to induce cell death is dependent upon the stage of follicle development and is related to the inherent susceptibility or resistance of granulosa cells to apoptosis. The present observations contribute to further establishing the relevance of the avian ovary as a useful model system in which to study cellular and molecular mechanisms mediating programmed cell death. Thus, ongoing investigations of the downstream signaling pathways involved in mediating the actions of ceramide will contribute to enhanced understanding of normal granulosa cell physiology and follicle atresia as well as provide insight regarding potential regulatory mechanisms that may influence the development of ovarian epithelial-derived tumors.

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