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Molecules and Cells

Induction of Apoptosis in Chicken Oviduct Cells by C2-Ceramide

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The chicken oviduct is a dynamic organ that produces secretory proteins such as ovalbumin and its cells undergo cell proliferation and differentiation. There has been no study of the cellular mechanism involved in cell death in the chicken oviduct. Therefore, this study has focused on the study of apoptosis in primary oviduct cells. Because ceramide is known to activate apoptosis in tumor cells and is produced in the oviduct, we used an exogenous ceramide analog to induce cell death. The viability of ceramide-treated chicken oviduct cells decreased in a dose-dependent manner and apoptotic cells were detected by staining with annexin V. The expression of apoptosis-related genes was assessed by RT-PCR and bcl-2 mRNA was found to decrease after exposure to ceramide while Bcl-x mRNA increased 12 h post-treatment. In addition, caspase-3 was expressed strongly in the early stages of apoptosis, while caspase-1 and -9 transcripts increased at later times. We conclude that ceramide induces apoptosis in oviduct-derived primary cells via a caspase- and bcl-2-dependent pathway.

Keywords: Apoptosis; Ceramide; Chicken Oviduct Cells.

Introduction

Ceramide, a key molecule in the sphingomyelin pathway, is produced by sphingomyelin hydrolysis, de novo synthesis, and breakdown of glycosphingolipid complexes. It induces apoptosis by altering mitochondrial function and recruiting a cascade of effector caspases (Kim *et al.*, 2003; Mizushima *et al.*, 1996; Smyth *et al.*, 1996). The diverse activities of ceramide involve different cell types, and a variety of signal receptors (Ruvolo, 2001).

Apoptosis is a ubiquitous form of cell death mediated by one or more cysteine proteases (caspases), among other intracellular factors. Caspase cascades are responsible for both initiating and amplifying early apoptotic signals (e.g. caspase-1, -2, -8, -9, -10) as well as "executing" apoptosis (e.g., caspase-3, -6, -9) (Kumar and Colussi, 1999; Nicholson and Thornberry, 1999). Other apoptosis-related genes encode members of the bcl-2 family that act at critical life-death decision points. Bcl-2 family members are classified in two categories. The Bcl-2 family consists of pro-apoptotic proteins such as Bax, Bok and Bak, while the death-suppressors consist of Bcl-x, Bcl-2 and Bcl-w (Gibson *et al.*, 1996; Metcalfe *et al.*, 1999). The anti-apoptotic proteins inhibit apoptosis by blocking the apoptotic actions of cytochrome c and apoptosis-inducing factor (AIF). Members of the Bcl-2 family are expressed to varying degrees in different cell types. Characterizing their profile in individual tissues and cells is therefore essential for understanding the molecular control of apoptosis in specific physiological contexts. Thus, for example, Bcl-2 and Bcl-x, as cell survival molecules, are highly expressed during chicken spermatogenesis (Vilagrasa *et al.*, 1997).

The chicken oviduct has been extensively used to study hormonal induction of protein synthesis. In response to steroid hormones the tubular gland cells undergo proliferation and differentiation and produce egg white proteins

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Abbreviations: AIF, apoptosis-inducing factor; DES, diethylstilbestrol; OD, optical density; pNA, p-nitroaniline; PS, phosphatidylserine; RT-PCR, reverse transcription-polymerase chain reaction.

(Jung-Testas *et al.*, 1986). After estrogen is withdrawn, the cells undergo apoptosis (Monroe *et al.*, 2000). The level of ceramide increases at that time and could be responsible for inducing the apoptosis (Tilly *et al.*, 1991; Witty *et al.*, 1996). In the present study, we examined the expression of Bcl-2 and caspase protein families in chicken oviduct cells exposed to ceramide.

Materials and Methods

Reagents All reagents were from Sigma unless otherwise specified. Synthetic C2-ceramide (N-acetyl-sphingosine) was dissolved in ethanol at a stock concentration of 20 mM and diluted into serum-free DMEM at the indicated concentrations. The final concentration of ethanol did not exceed 0.25%.

Isolation and primary culture of chicken oviduct cells Two-week-old White Leghorn chickens were implanted with a 15 mg diethylstilbestrol (DES) pellet for 14 d (primary stimulation) and the pellets then removed. After 28 days, two 15-mg DES pellets were re-implanted (secondary stimulation) for 2 weeks. The pellets were withdrawn and after 48 h the chickens were decapitated. The magnums of the oviducts were excised, trimmed of extraneous tissues, and minced with scissors into 1- to 2-mm pieces. The minced oviducts were incubated in dissociation medium: Ham's F-12 nutrient mixture, 250 U/ml crude collagenase, 250 μg/ml pronase, 20 μg/ml trypsin, 100 U/ml penicillin and 100 μg/ml streptomycin. The tissues were placed on a rotary shaker (200 rpm) at 37°C and mechanically disrupted by 10 passages through a 10-ml pipette over a period of 30 min. After dissociation, the fragments were allowed to settle for 2–3 min, and the supernatant was removed and discarded, and the remaining tissue was incubated in fresh dissociation medium for a further 30 min. After this second dissociation, the supernatant was transferred to 50-ml conical tubes, newborn calf serum was added to a final concentration of 10%, and the tubes were centrifuged at 600 rpm for 5 min. The cell pellet was resuspended in 30 ml of F12 medium containing 0.1% bovine serum albumin, and centrifuged again. The final cell pellet was resuspended in culture medium (DMEM, 10% fetal bovine serum and antibiotics) and plated in culture dishes. Because fibroblasts attach rapidly to the bottom of dishes, after 2 h incubation the epithelial cells remaining in suspension were removed gently and replated in other culture dishes in DMEM:F12 medium with 10^{-7} M of 17 beta-estradiol, 10^{-6} M corticosterone, and 50 ng/ml of insulin. The medium was changed every 24 h.

Trypan blue survival assay Cells were suspended in 0.4% trypan blue in PBS (pH 7.4), and 200 cells were counted. Cells that excluded the blue dye were scored as live. The percentage of live cells was plotted and averaged over at least three measurements.

Immunofluorescence staining Apoptotic cells were detected on the principle that Annexin V binds to translocated plasma membrane phosphatidylserine (PS). During the apoptotic process, PS translocates from the inner membrane to the outer membrane of the cells where it is accessible to Annexin V. The cells to be examined were fixed with 4% (w/v) paraformaldehyde-PBS for 15 min, permeabilized with 0.1% (v/v) NP-40 for 12 min, then blocked with 10% (w/v) horse serum in PBS for 30 min at room temperature. Annexin V-Cy3 (Sigma) was added for 45 min at room temperature. After washing with 0.1% NP-40/PBS, 0.1 μg/ml of 4′6′-diamidino-2-phenylindole (DAPI) was added to stain nuclei. Fluorescence images were taken with a fluorescence microscopy.

Isolation of total RNA Total RNA was isolated with TRIzol™ reagent (Gibco-BRL, USA) according to a modification of the manufacturer's instructions. Cultured cells in 10 mm plates were lysed with 1 ml of TRIzol™ and transferred to two 1.5 ml microtubes. Each tube was centrifuged at 14,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a fresh DEPC-treated tube, 500 μl of isopropyl alcohol was added and the tube centrifuged again at 14,000 rpm for 20 min. The pellet was washed with 1 ml of 75% ethanol, air-dried, and dissolved in DEPC-water. RNA content was calculated from the A_{260} value read with a UV-spectrophotometer (Amersham Pharmacia Biotech, Sweden).

Reverse transcription-polymerase chain reaction (RT-PCR) Reverse transcription was carried out using total RNA. The mixture containing 1 μg of total RNA, 10 μM of 3′ primers and deionized H_2O was incubated for 5 min at 65°C and then placed on ice for 3 min. One μl of dNTP mixture (10 mM each), 4 μl of 5× RT buffer, and 1 μl of MMLV reverse transcriptase (200 U/μl; Promega, USA) were added and incubation continued for 90 min at 42°C. To terminate the reaction the mixture was transferred for 5 min to 94°C, and placed on ice for 3 min. This single strand cDNA was stored at –20°C until used in the PCR reactions. The DNA was denatured for 2 min at 95°C prior to each PCR cycle (27–30 cycles), which consisted of 95°C for 15 s, annealing at 58°C for 1 min, 72°C for 1 min, followed by 7 min at 72°C before refrigeration. The primers employed are listed in Table 1. PCR reactions were performed with a GeneAmp PCR 2400 (Perkin-Elmer, USA). The entire contents of the reaction mixtures were separated by electrophoresis on 0.7% agarose gels, stained with ethidium bromide, and analyzed with a Geldoc system (Bio-rad, USA).

Assay of caspase activity Oviduct cells were treated with C2-ceramide for the indicated times, trypsinized, and washed with PBS. They were resuspended in ice-cold lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA) for 5 min and centrifuged at 10,000 × *g* for 10 min. Aliquots of the extracts were placed in a reaction buffer containing 2 mM substrate conjugated to p-nitroaniline (pNA) and incubated at 37°C for 2 h, Thereafter the optical density (OD) was measured at 405 nm.

Table 1. Primers for amplifying caspase and Bcl-2 family members.

Name of primer	DNA sequence (5′ → 3′)
Bcl-2 F	GGA GAA GAG GCT ACG ACA
Bcl-2 R	ATG TCC AAG ATA AGC GCC
Bok F	TGC AGA GGT GAT GGA GGT
Bok R	AAG AAG ATG GCC TTG AGG
Bcl-x F	TTC CTA CAA GCT CTC GCA
Bcl-x R	TTG TTG AAG GTC TCC TGG
Caspase 1 F	GAC CTT GTG CAT CAC CTC
Caspase 1 R	TCC TCT CTT GTG TTG GCA
Caspase 3 F	AAG ATG GAC CAC GCT CAG
Caspase 3 R	TGT CGA GTG GAG CAG GAT
Caspase 6 F	CGT GTT CAG TTG GAC AGC
Caspase 6 R	GAA CTC CAA GGA AGA GCC
Caspase 9 F	AAG GAG CAA GCA CGA CAG
Caspase 9 R	AGC AGG TCT TCA GAA CGG
GAPDH F	ATG GTG AAA GTC GGA GTC A
GAPDH R	ATC AAA GGT GGA GGA ATG G

* F, Forward primer; R, Reverse primer.

Results

Ceramide-mediated cell death in oviduct-derived cells C2-ceramide is a cell-permeable and biologically active form of ceramide. To determine its effect on cell viability, chicken oviduct cells were treated with C2-ceramide in a dose-dependent manner. As shown in Fig. 1A, after 12 h of treatment with ceramide, 50–70% of the chicken oviduct cells were dead. In 25 μM C2-ceramide, only 5% of the cells survived 24 h treatment.

To determine whether the cell death was apoptotic, we observed the morphology of the treated cells with a phase contrast microscopy (Fig. 1B). The cells were found to be rounded, shrunken and they often contained membrane blebs; in the absence of ceramide their morphology was normal. This ceramide-induced apoptosis was confirmed by means of the annexin V assay which detects an early stage of apoptosis. As shown in Fig. 2, annexin V stained the perinuclear membrane area of the C2-ceramide treated cells, whereas there was no staining in control cells. These results indicate that primary oviduct cells undergo apoptosis in response to C2-ceramide.

The level of Bcl-2 family mRNAs in the ceramide-treated oviduct cells To determine whether members of the Bcl-2 gene family are involved in the apoptotic program, we examined their expression in the ceramide-treated oviduct cells. Five μM C2-ceramide reduced the level of Bcl-2 mRNA in a time-dependent manner (Fig. 3). The expression of Bcl-x and Bok mRNA gradually increased with time.

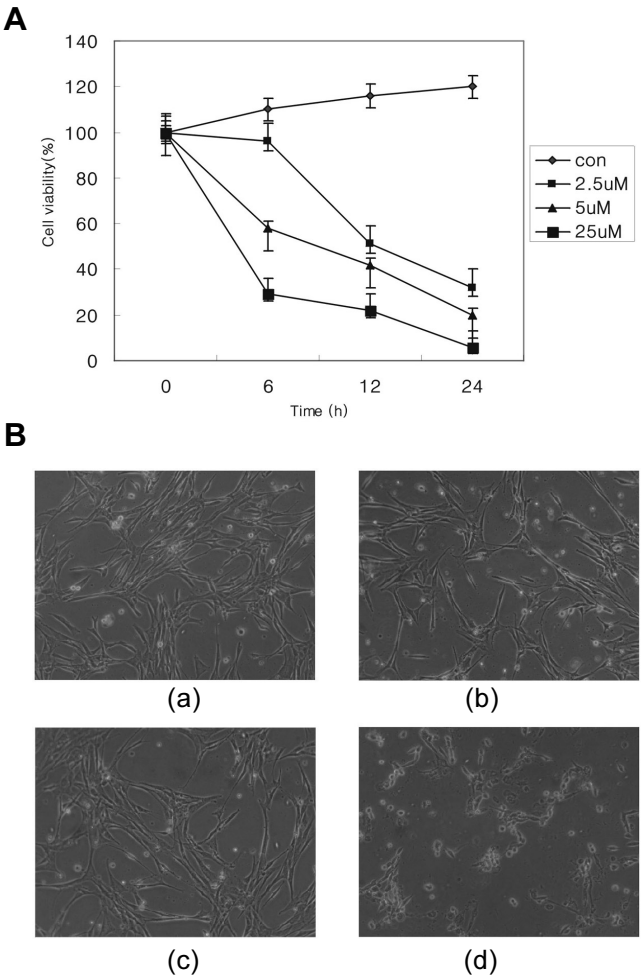

Fig. 1. Effects of C2-ceramide on cell viability and morphology in primary oviduct cells. **A.** Viability was measured in the presence of different concentration of C2-ceramide. Data are expressed as percentages of control values. **B.** Phase contrast photomicrographs of oviduct cells treated with ethanol vehicle (a), 2.5 μM C2-ceramide (b), 5 μM C2-ceramide (c), and 25 μM C2-ceramide (d) for 24 h. Note that some of the C2-ceramide-treated cells are dead, while others are rounded with membrane blebs.

The expression of caspase family members in the ceramide-treated cells The levels of caspase-1, -3, -6, and -9 mRNA were determined in oviduct cells exposed to C2-ceramide (Fig. 4A). After 3 h of ceramide treatment caspase-3 mRNA increased and maintained until 12 h. The expression of caspase-1 and -9 mRNAs increased gradually up to 12 h and returned to basal level after 24 h. We also found that caspase 3 was activated during the ceramide-induced apoptosis. As shown in Fig. 4B, the activity of this effector caspase began to increase after 3 h of ceramide-treatment and reached a peak by 12 h. These results indicate that caspase-dependent mechanisms are implicated in the ceramide-induced apoptosis.

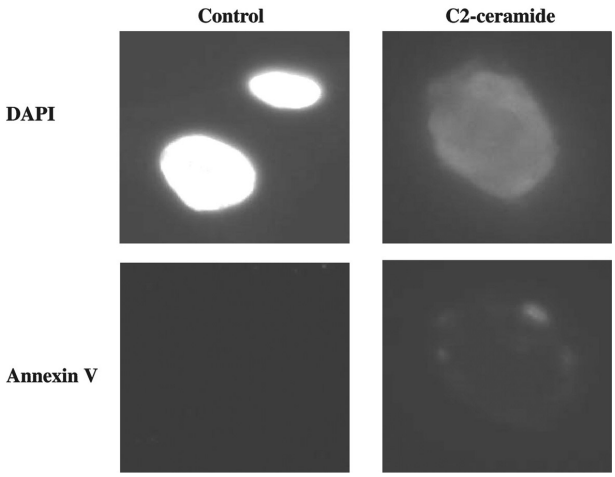

Fig. 2. The detection of annexin V in the ceramide-treated cells. Oviduct cells were stained with annexin V after exposure to 5 μM C2-ceramide for 24 h and the annexin V was detected by fluorescence microscopy. No signal was detectable in control cells, indicating the specificity of the staining.

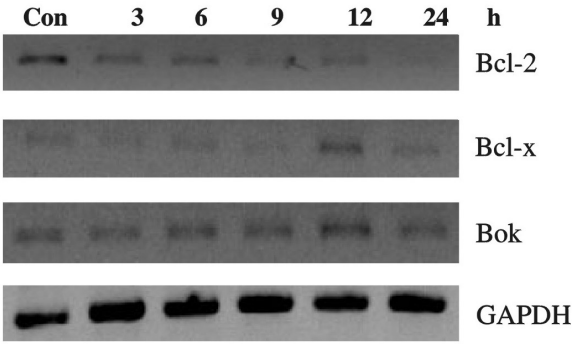

Fig. 3. Steady-state mRNA levels of Bcl-2 family members in the ceramide-treated cells. The expression of Bcl-2, Bcl-x and Bok gene was determined in oviduct cells treated with 5 μM C2-ceramide for the indicated times. GAPDH was used as loading control.

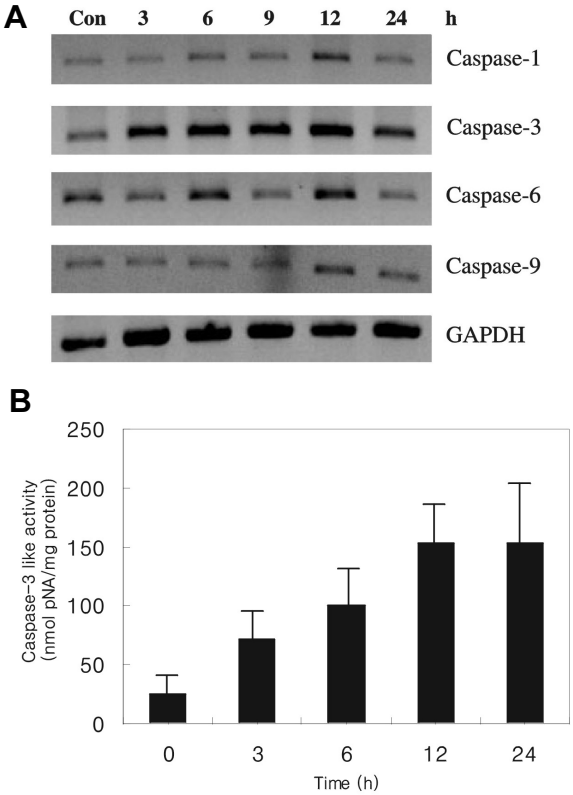

Fig. 4. The expression and activity of caspase family members during ceramide-induced cell death. **A.** The levels of caspase family mRNAs in oviduct cells were examined by RT-PCR in the presence of C2-ceramide. GAPDH data are shown to demonstrate equal loading. This experiment was repeated twice with similar results. **B.** Caspase activity was assayed in the ceramide-induced oviduct cells using substrates for caspase-3 such as (aspartate-glutamate-valine-aspartate).

Discussion

In the present study we used the avian ovary model to examine the mechanism of cell death in oviduct cells *in vitro*. It has been reported that apoptosis occurs in the early stages of oviduct regression in induced molting hens (Heryanto *et al.*, 1997; Jeong and Kim-Ha, 2003). Sex hormones in general have a protective effect against apoptosis in the oviduct (Thompson, 1994). Estrogen in particular has a protective role in chicken oviduct cells because these cells undergo apoptosis when deprived of estrogen (Monroe *et al.*, 2002).

Ceramide generated by sphingomyelin hydrolysis or synthase activity serves as a second messenger (Futerman and Hannun, 2004). Under appropriate circumstances increased levels of ceramide lead to terminal differentiation or apoptotic events in ovarian cells (Santana *et al.*, 1996; Tilly *et al.*, 1991; Witty *et al.*, 1996). Apoptotic cell death activated by tumor necrosis factor-alpha, Fas (APO-1/CD95) and UV is mediated by the sphingomyelin cycle (Hannun and Luberto., 2000; Kolesnick *et al.*, 1994; Verheij *et al.*, 1996). The role of ceramide as a second messenger in this process has been demonstrated by the finding that cell-permeable ceramide analogs (e.g. N-acetylsphingosine) are able to mimic the cell death-inducing effects of TNF-alpha, Fas, and UV (Ahn *et al.*, 1999; Kolesnick *et al.*, 2000; Venkataraman and Futerman, 2000).

Our results demonstrate that C2-ceramide induces apoptosis of chicken oviduct cells. In order to establish whether cell death following ceramide treatment involves necrosis or apoptosis, we stained the oviduct cells with annexin V. This confirmed that ceramide induces apoptosis. We observed down-regulation of *bcl-2* that is intimately involved in the control of apoptosis. Bcl-2 is an

anti-apoptotic gene and hence its down-regulation is associated with the initiation of apoptosis (Zhang *et al.*, 1997). The effect of ceramide on bcl-x and bok appeared to be less pronounced. Proteins of the bcl-2 family are important regulators of apoptosis (Zhang *et al.*, 1999); homodimers of death agonists induce apoptosis, while heterodimers or homo-dimers of death antagonists prevent apoptosis (Reed, 1999). Therefore, in the present study, apoptosis in ceramide-treated cells may be induced by the p53 activation in the suppression of bcl-2.

To obtain further insight into the mechanism of ceramide action, we studied the expression of caspase-3, a major player in apoptotic cell death. Caspases are activated during apoptosis and play critical roles in both the initiation and the execution of apoptosis (Cohen, 1997). Caspase-3 is essential for DNA fragmentation and the morphological changes associated with apoptosis (Janicke *et al.*, 1998). In the present study, expression of caspase-3 began to increase after 3 h of treatment. Caspase-3 was activated and annexin-V staining was evident in cells treated with ceramide, but we did not detect DNA fragmentation. This pattern is consistent with previous evidence that initiator caspases are expressed at a lower level than executioner caspases (Droin *et al.*, 2001; Genini *et al.*, 2001). Our results suggest that ceramide-induced apoptosis involves caspase-3 activation and a resultant cascade of reactions.

In summary, we have demonstrated that ceramide induces apoptosis in chicken oviduct cells. The induction of apoptosis by ceramide involves multiple pathways. Ceramide upregulates pro-apoptotic genes and down-regulates anti-apoptotic genes. Alteration of the balance of pro- and anti-apoptotic products in favor of apoptosis is one possible mechanism of ceramide-induced apoptosis in the chicken oviduct cells.

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