Role of prostaglandins in the suppression of apoptosis in hen granulosa cells by transforming growth factor α

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Although transforming growth factor α (TGF- α) is known to be an important survival factor for granulosa cells, the cellular and molecular mechanisms involved are uncertain. The purpose of the present study was to investigate the possible involvement of prostaglandins in the anti-apoptotic action of TGF- α . Hen granulosa cells from healthy prehierarchal follicles (2–6 mm) cultured in serum-free medium underwent spontaneous apoptosis as demonstrated by DNA fragmentation and nuclear chromatin condensation. TGF- α (20 ng ml⁻¹) stimulated maximum synthesis of prostaglandins (PGE and PGF) in granulosa cells and completely inhibited serum deprivation-induced apoptosis. The addition of an inhibitor of cyclooxygenase (COX; *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398) or ibuprofen) or phospholipase A₂ (PLA₂; aristolochic acid, 2-*p*-amylcinnamoyl amino-4-chlorobenzoic acid (ONO-RS-82) or arachidonyl trifluoro methyl ketone (TFMK)), to the

culture medium markedly suppressed the TGF- α -induced prostaglandin synthesis and significantly increased granulosa cell apoptosis. The apoptotic effect of NS398 and aristolochic acid was completely inhibited by exogenous prostaglandins (PGF_{2 α} , PGE₁, PGE₂) and arachidonic acid, respectively. However, exogenous prostaglandins failed to inhibit the PLA₂ inhibitor-induced apoptotic DNA fragmentation, implying that in addition to prostaglandins, arachidonic acid or leukotrienes may be important in transducing the anti-apoptotic action of TGF- α . In the absence of exogenous TGF- α , prostaglandins had no significant influence on granulosa cell apoptosis induced by serum withdrawal. These findings indicate that prostaglandin synthesis is a necessary, but not sufficient, event in the suppression of granulosa cell apoptosis by TGF- α . Whether arachidonic acid or leukotrienes are important in the anti-apoptotic action of TGF- α in hen granulosa cells remains to be determined.

Introduction

Ovarian follicular atresia is a degenerative process that involves granulosa cell apoptosis (Hughes and Gorospe, 1991; Tilly *et al.*, 1991b) and is controlled by gonadotrophins, steroids, cytokines and growth factors. During follicular development in the hen ovary, most of the follicles (> 99%) become atretic and regress before reaching 9 mm in diameter. Follicles that develop to > 9 mm in diameter are committed to ovulate and generally will not succumb to atresia (Johnson *et al.*, 1996). Although the control of follicular atresia by gonadotrophins is well documented (Hirshfield and Midgley, 1978; Chun *et al.*, 1996), less is known about its regulation by non-gonadotrophin signals. Since follicular atresia in hens normally occurs at the prehierarchal stage of follicular development (follicular size < 9 mm in diameter), when granulosa cells are much less responsive to FSH compared with those from hierarchical follicles (> 9 mm), the role of intraovarian local survival factors (for example, TGF- α) may be crucial in

determining the fate of prehierarchal follicles (atresia versus ovulation).

TGF- α is a close relative of epidermal growth factor (EGF), and exerts its physiological action through binding to EGF receptors. Although TGF- α receptor and its ligands (EGF and TGF- α) are present in the granulosa, theca interna and theca externa layers of the hen ovarian follicle throughout development, the highest concentrations are found in the smallest follicles (Onagbesan *et al.*, 1994), indicating a potential autocrine or paracrine role of TGF- α in the early stage of follicular development in the hen ovary. TGF- α is mitogenic in hen granulosa cells and prostaglandins mediate the actions of this growth factor in several types of cell (Harrison, 1994; Li and Tsang, 1994, 1995). Membrane phospholipids such as phosphatidylcholine are metabolized by cytosolic phospholipase A₂ (cPLA₂) to arachidonic acid and lysophosphatidic acid. Cyclooxygenase (COX) then converts arachidonic acid to prostaglandins. Alternatively, arachidonic acid can be metabolized to leukotrienes via the action of lipoxygenase. TGF- α activates cPLA₂ (Li *et al.*, 1997) and upregulates the expression of COX II mRNA and protein (Li *et al.*, 1996) in differentiated hen granulosa cells, resulting in increased

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prostaglandin synthesis (Li and Tsang, 1994, 1995). Although TGF- α attenuates serum deprivation-induced granulosa cell apoptosis in rats (Tilly *et al.*, 1992) and hens (Johnson *et al.*, 1996), it is not known whether prostaglandins mediate the anti-apoptotic action of TGF- α .

The present study investigated the possible role of prostaglandins in the suppression of granulosa cell apoptosis by TGF- α *in vitro*.

Materials and Methods

Reagents

QIAamp blood kit and QIAquick nucleotide removal kit were purchased from Qiagen Inc. (Chatsworth, CA). Arachidonyl trifluoromethyl ketone (TFMK), *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS398) and arachidonic acid were purchased from Cayman Chemical Company (Ann Arbor, MI). Culture media (minimum essential medium (MEM) and M199), culture media reagents (L-glutamine, non-essential amino acids, penicillin, streptomycin and fungizone) and fetal bovine serum (FBS) were purchased from Gibco/Bethesda Research Laboratories (Burlington, Ontario). Hydroxyethylpiperazine ethanesulfonic acid (Hepes) was obtained from BDH (Toronto, Ontario). Klenow polymerase was purchased from New England Biolabs (Beverly, MA). α -[32 P]dCTP (250 μ Ci per vial) was obtained from Amersham Life Science (Oakville, Ontario). Recombinant human transforming growth factor alpha (TGF- α) was purchased from Collaborative Research (Bedford, MA). 2-*p*-amylcinnamoyl amino-4-chlorobenzoic acid (ONO-RS-82), agarose, aristolochic acid and Hoescht 33258 dye were obtained from Sigma Chemical Co. (St Louis, MO). [5,6,8,9,11,12,14,15- 3 H(N)]-PGF $_{2\alpha}$ and [3,6,8,11,12,14,15- 3 H(N)]-PGE $_2$ (100–200 Ci per mmol) and scintillation fluid (ScintiVerse) were obtained from Dupont/NEN Research Products (Mississauga, Ontario). Charcoal, dextran, gelatin and eosin Y were purchased from Fisher Scientific (Nepean, ON). Prostaglandins (PGE $_1$, PGE $_2$ and PGF $_{2\alpha}$) were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). PGE $_2$ and PGF $_2$ antisera were gifts from N. R. Mason (Lilly Research Laboratories, Indianapolis, IN) and D. T. Armstrong (University of Western Ontario, London, Ontario), respectively.

Isolation and culture of granulosa cells

White Leghorn hens (Berry's Egg Farm, Kempville, ON) in their first year of egg laying were caged individually in an air conditioned (22°C) windowless room under a 14 h light:10 h dark photoperiod with free access to food and water. Hens were monitored to determine the pattern of egg laying and were killed approximately 10–13 h after egg laying. These studies were carried out in accordance with the Guides of the Canadian Council on Animal Care on the use and handling of experimental animals and approved by the Animal Care Committee of the Loeb Health Research

Institute. Ovaries were excised and placed in cold medium 199 supplemented with Hepes (25 mmol l $^{-1}$, pH 7.4). Granulosa cell layers from approximately 30 morphologically healthy prehierarchal follicles (round, not flaccid and yolk filled; 2–6 mm in diameter) were isolated, pooled and dissociated by treatment with medium 199 containing collagenase (270 U ml $^{-1}$) and trypsin inhibitor (0.01% w/v; 37°C, 5–10 min). Granulosa cell viability, as determined by the trypan blue exclusion test, was over 90%. Granulosa cells (1×10^6 per dish) were plated (6 h, 37°C) in 35 mm plastic dishes in 1 ml MEM (supplemented with L-glutamine (0.29 mg ml $^{-1}$), non-essential amino acids (0.1 mmol l $^{-1}$), streptomycin (100 μ g ml $^{-1}$) and penicillin (100 iu ml $^{-1}$)) containing 10% FBS under an atmosphere of 5% CO $_2$ and 95% air, and cultured for an additional 24 h in 1.5 ml of either fresh MEM with FBS (10%), or serum-free MEM containing various test agents (TGF- α , COX or PLA $_2$ inhibitors, prostaglandins or arachidonic acid). The change of media at the end of the plating period (6 h) also served to remove floating (or dead) cells, leaving 'healthy' cells tightly adherent to the culture surface. Thus, only viable undifferentiated granulosa cells from prehierarchal follicles were used. At the end of the culture period, media were collected and centrifuged (5 min, 10 000 g) to remove any floating cells. The cell-free media was supplemented with 2.0 ml absolute ethanol for prostaglandin assay, whereas the cells recovered after centrifugation as well as those attached to the culture dish were pooled and stored at –80°C, pending DNA extraction.

Prostaglandin measurements

Appropriate volumes of the culture media (in duplicates) were analysed for PGE and PGF by radioimmunoassay as described by Jaffe *et al.* (1973). PGE $_2$ and PGF $_{2\alpha}$ were used as standards. Since the antisera against PGE $_2$ and PGF $_{2\alpha}$ had significant crossreactivity against PGE $_1$ (16.8%) and PGF $_{1\alpha}$ (125.0%), respectively (Lewis *et al.*, 1978; Evans *et al.*, 1981), the data are reported as PGE and PGF equivalents. Prostaglandin production in each treatment group was normalized against total cell protein content, as determined by the Bio-Rad protein assay.

Biochemical assessment of apoptosis (DNA ladders)

DNA was isolated using the QIAamp blood kit following the manufacturer's instructions. Briefly, cells were lysed and subjected to RNase and proteinase K treatment. Samples were loaded on to spin columns containing silica membranes, which specifically bound DNA (80 bp to 50 kb in size) and DNA was eluted with Tris buffer (10 mmol l $^{-1}$, pH 9). DNA (500 ng) from each sample was radiolabelled with α -[32 P]dCTP, using the Klenow reaction as described by Rosl (1992) to quantify the extent of DNA fragmentation in cultured cells. Radiolabelled DNA was resolved by agarose (2%) gel electrophoresis in Tris-acetate EDTA buffer (40 mmol Tris l $^{-1}$, 20 mmol glacial acetic acid l $^{-1}$, 1 mmol EDTA l $^{-1}$) for about 150 min at 60–85 V. The gel was dried

and exposed to X-ray film or a phosphor screen for 0.5–24.0 h. The intensity of labelled low molecular mass (fragmented) DNA of 15 kb or less in each lane was determined using Molecular Dynamics Phosphorimager (Bio-Rad, Mississauga, ON).

Morphological assessment of apoptosis

At the end of the culture period, cells were detached from the culture surface by trypsin treatment (0.025 g ml⁻¹ in PBS; 37°C; 2 min) and combined with the floating cells from the medium. Cells were fixed with 37% formaldehyde (10%; v/v) and incubated with the nuclear stain Hoechst 33258 (2 µg ml⁻¹ in PBS; 10 min). An aliquot of the cell suspension was placed on a glass slide, viewed under UV light and photographed at a magnification of × 250. Cells with fragmented or highly condensed (brightly stained) nuclei were considered apoptotic. Between 75 and 220 cells were counted per treatment group in each experiment.

Statistical analysis

Data represent results from at least three experiments and were analysed statistically by one-way ANOVA. Significant differences between groups were determined by the Newman–Kuel's test. Statistical difference was inferred at $P < 0.05$.

Results

Effect of TGF- α on serum deprivation-induced apoptosis

Granulosa cells cultured in serum-free medium for 24 h showed spontaneous apoptotic DNA fragmentation, which was significantly suppressed by the presence of 10% FBS (Fig. 1a,b). TGF- α (5–100 ng ml⁻¹) also inhibited apoptotic DNA fragmentation in a concentration-dependent manner in the absence of FBS. Maximum inhibition was observed at a concentration of 20 ng TGF- α ml⁻¹, which decreased the serum deprivation-induced DNA fragmentation by 70% (Fig. 1a,b) and significantly reduced the proportion of apoptotic granulosa cells (18% under serum deprivation versus 4% in the presence of TGF- α ; Fig. 2c). The ID₅₀ value for TGF- α in the suppression of DNA fragmentation (induced by serum withdrawal) was approximately 5 ng ml⁻¹ (Fig. 1b).

Effect of TGF- α on prostaglandin production

If prostaglandins mediate the action of TGF- α in granulosa cells, prostaglandin synthesis would be expected to be stimulated by TGF- α . Granulosa cells secreted appreciable amounts of PGE (12.9 ± 1.9 pmol per 10⁶ cells) and PGF (39.6 ± 3.3 pmol per 10⁶ cells) during a 24 h culture period in serum-free medium containing no exogenous TGF- α . The addition of TGF- α (5–100 ng ml⁻¹) to the cell cultures significantly ($P < 0.01$) increased both PGE and PGF secretion in a concentration-dependent manner (Fig. 1c). Maximum stimulation was observed at

Fig. 1. Suppression of apoptosis induced by serum deprivation and stimulation of prostaglandin synthesis in hen granulosa cells by transforming growth factor α (TGF- α) *in vitro*. Granulosa cells were cultured for 24 h in serum-free media containing 0, 5, 10, 20, 50 or 100 ng TGF- α ml⁻¹ (b; ■) or in media containing only 10% fetal bovine serum (FBS) (b; □). DNA was isolated, radiolabelled with [α ³²P]dCTP and resolved by agarose gel electrophoresis. (a) Representative autoradiogram showing apoptotic DNA ladders. (b) Densitometric quantification of the low molecular weight DNA (≤ 15 kb), corresponding to the DNA ladders (a). Data are expressed as mean ($\pm$ SEM) percentage of control (in the absence of TGF- α); $n = 4$ –5 experiments. (c) Prostaglandin secretion in response to TGF- α , as determined by radioimmunoassay. PGE (■) and PGF (▲) secretion in the control group (in the absence of TGF- α ; defined as 1) was 12.9 ± 1.9 pmol per 10⁶ cells and 39.6 ± 3.3 pmol per 10⁶ cells, respectively. Data are expressed as mean ($\pm$ SEM) fold increase over control; $n = 9$ (PGE) and $n = 6$ (PGF) experiments. ** $P < 0.01$ versus control; *** $P < 0.001$ versus control.

20 ng ml⁻¹, which increased the secretion of PGE and PGF to 2.5- and 4.5-fold above that of the control, respectively. The ED₅₀ value for TGF- α in both PGE and PGF secretion was 5 ng ml⁻¹, which was similar to its ID₅₀ value for inhibition of serum deprivation-induced DNA fragmentation (Fig. 1b,c).

Effect of PLA₂ and COX inhibitors on TGF- α -induced prostaglandin production and suppression of apoptosis

COX and PLA₂ inhibitors were used to block prostaglandin synthesis to determine whether TGF- α -induced prostaglandin synthesis mediates the anti-apoptotic action of TGF- α . Granulosa cells were cultured for 24 h in serum-free media containing TGF- α (20 ng ml⁻¹) and either a COX inhibitor (NS398 (5–10 μ mol l⁻¹), ibuprofen (50–100 μ mol l⁻¹) or indomethacin (12.5–25.0 μ mol l⁻¹)) or a PLA₂ inhibitor (aristolochic acid (20–30 μ mol l⁻¹), TFMK (5–10 μ mol l⁻¹), or ONO-RS-82 (5–10 μ mol l⁻¹)). The COX inhibitors NS398 and ibuprofen, but not indomethacin, effectively attenuated the TGF- α -induced suppression of granulosa cell apoptosis (Fig. 3a,c,e). NS398 (5 μ mol l⁻¹) significantly increased (180%; $P < 0.05$) apoptotic DNA fragmentation compared with control cells (Fig. 3), and a concentration-dependent effect of the inhibitor was observed when a lower concentration range of the inhibitor was used (0, 1 and 10 μ mol l⁻¹; Fig. 2c) in assessment of nuclear morphology (proportion of apoptotic cells: 4 $\pm$ 0.2% (control), 8 $\pm$ 0.4% (1 μ mol l⁻¹ NS398), 16 $\pm$ 1.9% (10 μ mol l⁻¹ NS398)). All three PLA₂ inhibitors significantly induced, in a concentration-dependent manner and with various degrees of effectiveness, more extensive apoptotic DNA fragmentation in granulosa cells compared with TGF- α -treated control cultures containing no inhibitor (Fig. 3b,d,f). The addition of each COX inhibitor to the cell cultures, even at its lowest concentration, caused complete (approximately 100%) inhibition of prostaglandin synthesis ($P < 0.001$; Fig. 4a,c,e). The addition of PLA₂ inhibitors to the cell cultures also significantly ($P < 0.001$) suppressed the TGF- α -induced prostaglandin secretion (Fig. 4b,d,f). Aristolochic acid suppressed PGE and PGF secretion in a concentration-dependent manner: at 20 μ mol aristolochic acid l⁻¹ 35 and 55% inhibition, respectively, was noted. TFMK and ONO-RS-82, at concentrations as low as 5 μ mol l⁻¹, inhibited the secretion of both prostaglandins to a similar extent and elicited maximum inhibition of PGE and PGF secretion (75 and 82%, respectively).

Effect of exogenous prostaglandins and arachidonic acid on the apoptotic effect of COX (NS398) and PLA₂ (aristolochic acid) inhibitors

The ability of exogenous prostaglandins and arachidonic acid to prevent the effect of NS398 and aristolochic acid, respectively, was studied to determine whether inhibitor-induced DNA fragmentation was a consequence of non-specific effects of the inhibitors. PGE₁, PGE₂ or PGF_{2 α} (1 or 20 μ mol l⁻¹) effectively inhibited NS398 (10 μ mol l⁻¹)-

induced apoptotic DNA fragmentation (Fig. 2a,b) and, PGF_{2 α} (20 μ mol l⁻¹) also inhibited the apoptotic changes in nuclear morphology (Fig. 2c). These data confirmed that the effect of NS398 was related to its inhibition of prostaglandin synthesis, and that the anti-apoptotic action of TGF- α may be mediated by prostaglandins. Similarly, the presence of arachidonic acid (0.5 μ mol l⁻¹) completely inhibited the aristolochic acid (30 μ mol l⁻¹)-induced apoptotic DNA fragmentation ($P < 0.01$; Fig. 5a,b). In contrast, exogenous PGE₁, PGE₂ or PGF_{2 α} , at concentrations that effectively attenuated the COX inhibitor-induced apoptosis (1 or 20 μ mol l⁻¹), failed to inhibit the aristolochic acid-induced DNA fragmentation (Fig. 6a,b).

Potential of prostaglandins and arachidonic acid to mimic the anti-apoptotic action of TGF- α

Granulosa cells were cultured in serum-free medium in the presence or absence of PGF_{2 α} (20.0 μ mol l⁻¹), PGE₁ (20.0 μ mol l⁻¹), PGE₂ (20.0 μ mol l⁻¹), arachidonic acid (0.5 μ mol l⁻¹) or TGF- α (20.0 ng ml⁻¹) to determine whether prostaglandins or arachidonic acid alone could mimic the anti-apoptotic action of TGF- α . Compared with serum deprivation-induced DNA fragmentation (100%), the presence of PGF_{2 α} , PGE₁, PGE₂ or arachidonic acid did not significantly affect the extent of apoptotic DNA fragmentation (118 $\pm$ 13%, 103 $\pm$ 7%, 102 $\pm$ 12%, 114 $\pm$ 15%, respectively; $n = 4$ experiments).

Discussion

Hen granulosa cells from prehierarchical follicles (2–6 mm) cultured in serum-free medium underwent spontaneous apoptosis, as demonstrated by apoptotic DNA ladders and nuclear condensation. These results are in agreement with other reports of apoptosis induced by serum deprivation in hen granulosa cells from 4–8 mm follicles (Flaws *et al.*, 1995), gonadotrophin-treated rat granulosa cells (Tilly *et al.*, 1992), and several non-ovarian types of cell (Raff, 1992; Thompson, 1995). Apoptosis induced by serum- or survival factor-deprivation *in vitro*, as observed in undifferentiated hen granulosa cells, indicates that these cells must be under the constant 'protective' influence of anti-apoptotic factors *in vivo*. When these cell survival factors are 'removed' *in vitro*, the tonic suppression of apoptosis is no longer present and, as a result, apoptosis occurs. In this context, the addition of TGF- α to granulosa cell cultures was demonstrated to inhibit the serum deprivation-induced granulosa cell apoptosis effectively, which is consistent with other reports on the suppression of apoptosis by EGF and TGF- α (Tilly *et al.*, 1992; Johnson *et al.*, 1996; Laufey *et al.*, 1996; Reinartz *et al.*, 1996; Janz and Van der kraak, 1997). Theca and granulosa cells of hen ovarian follicles express the highest concentrations of both TGF- α and its receptor during the prehierarchical stage of development (Onagbesan *et al.*, 1994), indicating an autocrine or paracrine mode of action for TGF- α . The role of TGF- α as a

Fig. 2. Prevention of the 2-cyclohexyloxy-4-nitrophenyl methanesulfonamide (NS398)-induced apoptosis in hen granulosa cells by exogenous prostaglandins during a 24 h culture period. (a) Representative autoradiogram showing apoptotic DNA ladders. (b) Densitometric quantifications of the low molecular weight DNA (≤ 15 kb); corresponding histograms positioned directly below their DNA ladders in (a). Control (in the absence of prostaglandin but in the presence of transforming growth factor α (TGF- α) (20 ng ml $^{-1}$) and NS398 (10 μ mol l $^{-1}$)) is defined as 100%. Data are expressed as mean ($\pm$ SEM) percentage of control; $n = 3$ experiments. * $P < 0.05$ versus control; ** $P < 0.01$ versus control; *** $P < 0.001$ versus control. (c) The percentage of apoptotic cells as determined by nuclear morphology using Hoechst 33258. Granulosa cells were cultured in serum-free medium or serum-free medium containing the indicated test agents. Cells were stained with Hoechst 33258 and the percentage of apoptotic cells was determined by assessing nuclear morphology. Data are expressed as mean ($\pm$ SEM); $n = 3$ –5 experiments. * $P < 0.05$ versus TGF- α alone; *** $P < 0.001$ versus TGF- α alone; † $P < 0.001$ versus TGF- α plus NS398 (1 μ mol l $^{-1}$); ‡ $P < 0.001$ versus TGF- α plus NS398 (10 μ mol l $^{-1}$).

Fig. 3. Induction of apoptotic DNA fragmentation in hen granulosa cells by cyclooxygenase (COX) inhibitors (a,c,e) and phospholipase A₂ (PLA₂) (b,d,f) inhibitors in the presence of transforming growth factor α (TGF- α) (20 ng ml⁻¹) during a 24 h culture period. Cox inhibitors: (a) 2-cyclohexyloxy-4-nitrophenyl methanesulfonamide (NS398), (c) ibuprofen, (e) indomethacin. PLA₂ inhibitors: (b) aristolochic acid, (d) arachidonyl trifluoro methyl ketone (TFMK), (f) 2-*p*-amylcinnamoyl amino-4-chlorobenzoic acid (ONO-RS-82). Histograms show densitometric quantification of the low molecular weight DNA (≤ 15 kb). Control (20 ng TGF- α ml⁻¹; no inhibitor) is defined as 100%. Data are expressed as mean ($\pm$ SEM) percentage of control; $n = 3-7$ experiments. * $P < 0.05$ versus control; ** $P < 0.01$ versus control.

survival factor may be crucial at the prehierarchical stage of follicular development, as the undifferentiated granulosa cells from these follicles are much less responsive to gonadotrophins compared with differentiated granulosa cells from hierarchical and preovulatory hen follicles (Tilly *et al.*, 1991a). However, up to 4% of the cultured granulosa cells were apoptotic even in the presence of the concentration of TGF- α that produced the maximum effect

(20 ng ml⁻¹). This finding may be due to the inability of TGF- α *in vitro* to emulate the situation *in vivo* in which multiple apoptosis inhibitory (survival) factors may be impinging on the granulosa cells simultaneously. Alternatively, it is possible that such cells have already received a death signal and are destined to die, irrespective of the presence of survival factors *in vivo*.

Prostaglandins mediate the action of TGF- α in numerous

Fig. 4. Suppression of the transforming growth factor α (TGF- α)-induced prostaglandin secretion in hen granulosa cells by cyclooxygenase (COX) inhibitors (a,c,e) and phospholipase A₂ (PLA₂) inhibitors (b,d,f) during a 24 h culture period. Cox inhibitors: (a) 2-cyclohexyloxy-4-nitrophenyl methanesulfonamide (NS398), (c) ibuprofen, (e) indomethacin. PLA₂ inhibitors: (b) aristolochic acid, (d) arachidonyl trifluoro methyl ketone (TFMK), (f) 2-*p*-amylcinnamoyl amino-4-chlorobenzoic acid (ONO-RS-82). Prostaglandins secreted into the culture media were determined by radioimmunoassay. PGE and PGF secretion in the control group (20 ng TGF- α ml⁻¹, no inhibitor; defined as 100%) was 30.0 ± 3.8 pmol per 10⁶ cells and 185.1 ± 22.5 pmol per 10⁶ cells, respectively. Data are expressed as mean ($\pm$ SEM) percentage of control; $n = 5-8$ experiments. $P < 0.001$ for each data point versus respective control.

Fig. 5. Inhibition of the aristolochic acid-induced apoptosis in hen granulosa cells by arachidonic acid during a 24 h culture period. (a) Representative autoradiogram showing apoptotic DNA ladders. (b) Densitometric quantification of the low molecular weight DNA (≤ 15 kb); corresponding histograms for DNA ladder in (a). Control (transforming growth factor α (TGF- α) plus aristolochic acid; no exogenous arachidonic acid (a)) is defined as 100%. Data are expressed as mean ($\pm$ SEM) percentage of control; $n = 4$ experiments. ** $P < 0.01$ versus control.

physiological systems, including stimulation of DNA synthesis in differentiated hen granulosa cells (Li and Tsang, 1995) and in rat hepatocytes (Skouteris and McMenamin, 1992). However, it is unknown whether prostaglandins mediate the anti-apoptotic action of TGF- α in undifferentiated hen granulosa cells. In the present study, TGF- α (20 ng ml⁻¹)

significantly increased the secretion of PGE and PGF in granulosa cells and suppressed changes induced by serum deprivation in cellular and nuclear morphology and DNA fragmentation characteristic of apoptosis. These observations are consistent with the concept that prostaglandins mediate the TGF- α -induced suppression of granulosa cell apoptosis *in vitro*. Moreover, the addition of inhibitors of either COX or PLA₂ to the culture media markedly suppressed the TGF- α -induced prostaglandin synthesis and induced extensive granulosa cell apoptosis compared with TGF- α -treated control cultures. The effect of the COX inhibitors was related to the suppression of prostaglandin synthesis and was not due to cytotoxicity, as exogenous prostaglandins (PGF_{2 α} , PGE₁, PGE₂) were capable of inhibiting the apoptotic action of the inhibitor. These data provide evidence for the involvement of prostaglandins in the anti-apoptotic action of TGF- α .

Although arachidonic acid also inhibited granulosa cell apoptosis induced by the PLA₂ inhibitor aristolochic acid, exogenous PGE₁, PGE₂ or PGF_{2 α} at concentrations that effectively attenuated cell death induced by COX inhibitor (1 or 20 μ mol l⁻¹) were ineffective in inhibiting the aristolochic acid-induced apoptotic DNA fragmentation. It is suggested that prostaglandins are necessary (as demonstrated in studies with COX inhibitors) but not sufficient for mediating the anti-apoptotic action of TGF- α . It is possible that, in addition to prostaglandins, arachidonic acid and its metabolites (that is, leukotrienes) may be important for the anti-apoptotic action of TGF- α in hen granulosa cells. In this context it should be noted that both prostaglandins and leukotrienes are produced after EGF stimulation in A431 cells and appear to be important in the physiological function of the growth factor (Peppelenbosch *et al.*, 1993).

The mechanism by which prostaglandins suppress granulosa cell apoptosis in response to TGF- α is not known. It is possible that secreted prostaglandins may act via G-protein coupled receptors (that is, EP and FP receptor subtypes) on granulosa cell membranes. However, as 8-bromo-cAMP and vasoactive intestinal peptide (known to act via the protein kinase A (PKA) pathway) prevent apoptosis induced by serum deprivation in undifferentiated hen granulosa cells (Flaws *et al.*, 1995), it is unlikely that receptors primarily associated with the increase of intracellular cAMP (that is, EP₂ and EP₄ receptors) are involved in the action of PGF or PGE produced in response to TGF- α . This notion is consistent with findings from the present study showing that prostaglandins alone (in the absence of TGF- α or serum) neither suppress nor enhance granulosa cell apoptosis induced by serum deprivation. Hence, prostaglandins require the presence of other, as yet undefined, factors downstream of TGF- α receptor activation to produce the anti-apoptotic action of TGF- α . In granulosa cells, TGF- α and EGF exert their action via tyrosine kinase-dependent pathways (Tilly *et al.*, 1992; Keel *et al.*, 1995), mitogen-activated protein (MAP) kinase (Keel *et al.*, 1995), protein kinase C and Na⁺/H⁺ antiporter (Li *et al.*, 1991). Whether any of these TGF- α -induced signals co-

Fig. 6. Effect of prostaglandins on the aristolochic acid-induced apoptosis in hen granulosa cells during a 24 h culture period. (a) Representative autoradiogram showing apoptotic DNA ladders. (b) Densitometric quantification of the low molecular weight DNA (≤ 15 kb); corresponding histograms for DNA ladder in (a). Control (transforming growth factor α (TGF- α) plus aristolochic acid; no exogenous prostaglandins (a)) is defined as 100%. Data are expressed as mean ($\pm$ SEM) percentage of control; $n = 4$ experiments. * $P < 0.05$ versus control.

operate with prostaglandins to effect the TGF- α -mediated suppression of apoptosis is unknown. Preliminary studies from this laboratory have demonstrated that EP₃ receptors, known to show high and similar affinity for PGE₂ and PGE₁ but low affinity for PGF_{2 α} , are present in hen granulosa cells (J. Li and B. K. Tsang, unpublished). EP₃ receptor activation decreases cAMP concentrations by inhibiting adenylate cyclase (Negishi *et al.*, 1995). Although human and bovine granulosa cells express FP and EP₃ receptors (Tsai *et al.*, 1996; Ristimaki *et al.*, 1997), it is not known whether

hen granulosa cells also express FP or EP₁ receptors. Alternatively, prostaglandins may exert their effect in part via the peroxisome proliferator-activated receptor, a nuclear receptor that is activated by various fatty acids, including leukotriene B₄ and prostaglandins of the A, D and J series (Yu *et al.*, 1995; Devchand *et al.*, 1996). Peroxisome proliferator-activated receptor is a transcription regulator that maintains lipid homeostasis in the liver, promotes adipocyte differentiation and controls the duration of the inflammatory response in the liver (Devchand *et al.*, 1996;

Shalev and Meier, 1996; Wolf, 1996). It is unknown whether granulosa cells express this receptor.

In summary, granulosa cell viability *in vitro* is dependent on the continual supply of survival and growth factors (for example, serum), and removal of such factors spontaneously induces apoptosis. In addition, the present study shows that TGF- α is a survival factor for undifferentiated hen granulosa cells, as demonstrated by the capacity of the growth factor to suppress serum deprivation-induced apoptosis. Prostaglandin synthesis is a necessary, but not sufficient, event in the suppression of granulosa cell apoptosis by TGF- α . Whether arachidonic acid or leukotrienes are important in the anti-apoptotic action of TGF- α remains to be determined. It is possible that pre-hierarchical hen ovarian follicles need a continual supply of survival factors (such as TGF- α) to maintain their viability and protect them from atresia. The availability of survival factors or the capacity of granulosa cells to respond to them may be important determinants of the fate of hen pre-hierarchical ovarian follicles (atresia versus ovulation).

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