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Expression of Cytochrome *P*450 1A1, an Estrogen Hydroxylase, in Ovarian Granulosa Cells is Developmentally Regulated

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In this paper we report the analysis of porcine ovarian granulosa cells for the expression of several known hepatic estrogen hydroxylase RNAs. Of the *P*450s examined, only CYP 1A1 RNA was detected. Accordingly, the regulation of this mRNA was studied. The RNA for CYP 1A1 was dramatically and completely induced within 2 hours after exposure of immortalized granulosa cells to 3-methylcholanthrene (3MC) and expression could be inhibited with 10 μM phorbol myristate acetate. This message was also inducible by 3MC in cultured primary granulosa cells isolated from immature and developing follicles. Dexamethasone increased the relative expression of CYP 1A1 RNA in 3MC treated cells. In the absence of 3MC, the CYP 1A1 message was expressed in cultured granulosa cells from developing but not immature follicles, indicating developmental regulation of this enzyme. Further support for developmental regulation was provided by studies which detected the appearance of CYP 1A1 RNA during growth of ovarian follicles *in vivo*. This is the first report identifying a specific *P*450 estrogen hydroxylase RNA in ovarian granulosa cells.

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INTRODUCTION

While the liver is considered the primary site of estrogen (E_2) hydroxylation, other tissues, including the ovary, possess this activity [1–3]. Many of the E_2 hydroxylases identified to date are members of several well characterized *P*450s belonging to diverse families. These include members of the 1A, 2B and 2C, 3A and 4B families [3–5]. The level of some of these *P*450s is increased by treating animals or cultured cells with the appropriate endogenous or exogenous stimulant. The most common reason for this increase in protein content is enhanced gene transcriptional activity, but other mechanisms, such as increased RNA stability or translational activity have been reported [6].

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Abbreviations: E_2, 17β-estradiol; 3MC, 3-methylcholanthrene; Ah receptor, aryl hydrocarbon receptor; TPA, phorbol myristate acetate; CYP 1A1, cytochrome *P*450 1A1; PBS, phosphate buffered saline; DMEM, Dulbecco's Modified Eagles Medium; FSH, follicle stimulating hormone; PKA and PKC, protein kinase A and C.

Little is known about the role of E_2 hydroxylation at the cellular level. However, catecholestrogens (2- or 4-hydroxyestradiol) have been shown to stimulate progesterone biosynthesis by mechanisms independent of E_2 effects in ovarian granulosa cells [2]. Evidence also exists that E_2 hydroxylation in MCF7 cells (a human breast cancer cell line) may regulate the intracellular concentration of E_2, thereby interfering with E_2-dependent foci growth and development [7]. In these cells the E_2 hydroxylase was identified as *P*450 1A1, with hydroxylase activity only present in cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ligand for the Ah receptor [8] and transcriptional activator of the *P*450 1A1 gene complex. Despite these data in breast cancer cells, the enzyme(s) involved in extrahepatic E_2 hydroxylation have not been conclusively identified.

In order to better understand the role of E_2 metabolism in ovarian physiology, and the role such metabolites may play in cellular function, we analyzed primary and immortalized granulosa cells for the existence and expression of RNAs for several cytochrome

$P450$ E_2 hydroxylases. Our findings indicate that one $P450$ RNA, CYP 1A1 RNA, is expressed in porcine ovarian granulosa cells treated with 3MC, an Ah receptor ligand. In addition, granulosa cells expressed this RNA in the absence of an exogenous ligand for the Ah receptor in a developmentally-specific manner.

MATERIALS AND METHODS

Cell culture

The MDG2.1 cell line was derived from porcine ovarian granulosa cells (3–5 mm in dia.) by SV40 large T antigen immortalization [9]. For primary cell cultures, porcine ovaries were obtained at a local abattoir and granulosa cells collected from immature (1–3 mm) or developing (3–5 mm dia.) follicles as described previously [10]. These granulosa cells differ in morphological and biochemical characteristics; for example, cells from immature follicles are spindle shaped, secrete less progesterone, and are less gonadotropin responsive than those from developing (3–5 mm) follicles [10]. All granulosa cells were cultured in Complete Growth Medium [9, 11] (DMEM/F12, 5% fetal calf serum (FCS)/5% horse serum (HS), 1% UltraSerG (BRL; Bethesda Research Laboratories), 5 ng/ml transferrin, 3 U/ml insulin, 100 nM β-estradiol, 1 μM α-tocopherol, 1 μM dexamethasone, and 10 μM selenium) in a 5% humidified CO_2 atmosphere at 5×10^6 viable cells/60 mm dish.

RNA isolation from cultured cells and Northern blots

MDG2.1 cells were plated at 5×10^6 cells/60 mm culture dish for 24 h, washed with PBS, and the media changed to DMEM for the experimental time period. Primary cells were plated at the same initial density but allowed to grow for 5 days prior to RNA isolation, with a medium change at day 3. All cells were confluent at harvest. Total RNA was isolated from cultured cells by acid guanidinium thiocyanate:phenol–chloroform extraction after *in situ* cellular lysis [12]. The final RNA pellet was dissolved in 50–100 μl sterile water and RNA concentrations were determined spectrophotometrically at 260 nm. Ten μg of RNA was dried under vacuum, redissolved in 10 μl denaturing buffer (50% formamide/2.2 M formaldehyde/1 × SSC (1 × SSC is 150 mM NaCl/15 mM sodium citrate, pH 7), and denatured at 65°C for 10 min. Total RNA was separated by electrophoresis on 1% agarose/2.2 M formaldehyde gels for 3–4 h at 50 V [13]. After electrophoresis the migration of the ribosomal RNA was visualized by ethidium bromide (added to the loading buffer prior to electrophoresis) fluorescence under UV light and the position of the bands marked. The RNA was transferred from the gel to nylon membrane (Biodyne, ICN), baked at 80°C *in vacuo*, prehybridized for 4–6 h in probe-free hybridization solution, and hybridized in 50% formamide, 5 × SSCP (1 × is 120 mM NaCl, 15 mM sodium citrate, 13 mM KH_2PO_4, 1 mM EDTA, pH 7), 1% sodium dodecyl sulfate (SDS), 1% Denhardt's solution, and 5×10^6 cpm/ml radiolabeled probe at 42°C for 20–24 h. Membranes were washed 4 × at room temperature in 2 × SSC/0.5% SDS and twice at 50°C in 0.5 × SSC/0.5% SDS and exposed to film. Complementary DNA insert probes used in this analysis were obtained by restriction digestion of plasmid DNA (Bam H1 for pSV3neo, the source of the SV40 large T antigen probe; Eco R1 for CYP 1A1; Eco R1 and HincII for CYP 1A2; and Bam H1 for CYP 3A4) followed by agarose gel purification of the inserts. For β-actin RNA, the complete plasmid with cDNA insert was radiolabeled. All radioactive probes were generated by nick translation using a kit according to manufacturer's instructions (BRL). Intensity of autoradiographic signal of films or blots was quantitated with a Molecular Dynamics Laser Scanner Densitometer or a Betagen Scanner. Statistical analysis was performed using the Instat Statistical software package and Dunnett's multiple comparisons test. Representative data from a single experiment are depicted. In each instance, results were replicated in a separate experiment.

RNA isolation from cycling pig follicles and dot blots

The synthetic progestin altrenogest was added as a top feed for pigs to block follicle maturation as described previously [14]. Briefly, the ovaries were collected from the pigs at slaughter at 1, 3, 5, and 7 days after altrenogest withdrawal. Individual follicles were dissected out, homogenized with 4 M guanidinium isothiocyanate, and the RNA isolated by cesium chloride gradient ultracentrifugation. Following phenol–chloroform extraction, the RNA was precipitated with ethanol, redissolved in water, quantitated by optical density at 260 nm, and stored frozen at −70°C until used.

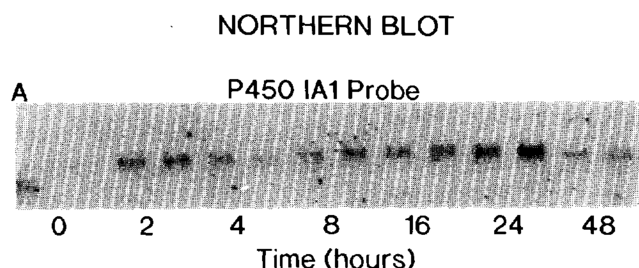

Fig. 1. Time course of induction of CYP 1A1 RNA in MDG2.1 ovarian granulosa cells. Cells were plated in duplicate at 5×10^6/60 mm dish for 24 h in complete growth medium and the medium changed to DMEM ± 5 μM 3MC. RNA was isolated at the times indicated (h). Ten μg total RNA from each dish was fractionated on agarose-formaldehyde gels and transferred to nylon membrane. Northern blots were probed sequentially with radiolabeled CYP 1A1 and β-actin cDNAs. Autoradiography was for 48 h for CYP 1A1 and 18 h for β-actin.

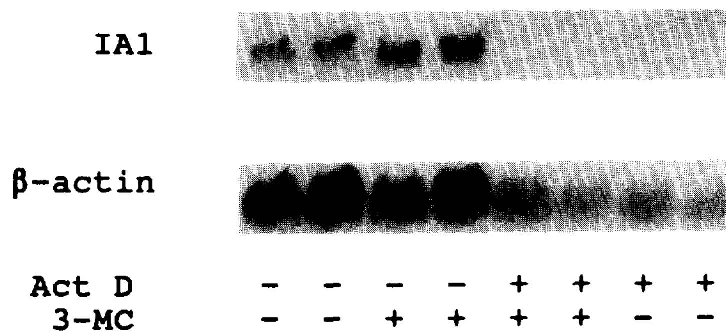

Fig. 2. Effect of actinomycin D on the expression of CYP 1A1 and β-actin RNAs. Northern blots were performed as described in Fig. 1. Cells were plated at 2×10^6 cells/60 mm dish for 36 h in growth medium, washed with PBS, and incubated in DMEM for an additional 4 h with actinomycin D (5 μg/ml) and/or 3MC (5 μM) as indicated. Autoradiography was for 3 days for CYP 1A1 and 3 h for β-actin.

For dot blots, 8 μg of RNA was aliquoted into individual microfuge tubes, dried under vacuum, redissolved in 100 μl denaturing buffer (described above), and denatured at 60°C for 10 min. After chilling on ice, 540 μl 20 × SSC was added to each tube, and 200 μl (for 2.5 μg) and 400 μl of denatured RNA was applied to nylon membrane using adjacent wells of the dot blot apparatus. Wells were rinsed with 400 μl 10 × SSC. The nylon membrane was probed for CYP 1A1 RNA as described for Northern blots.

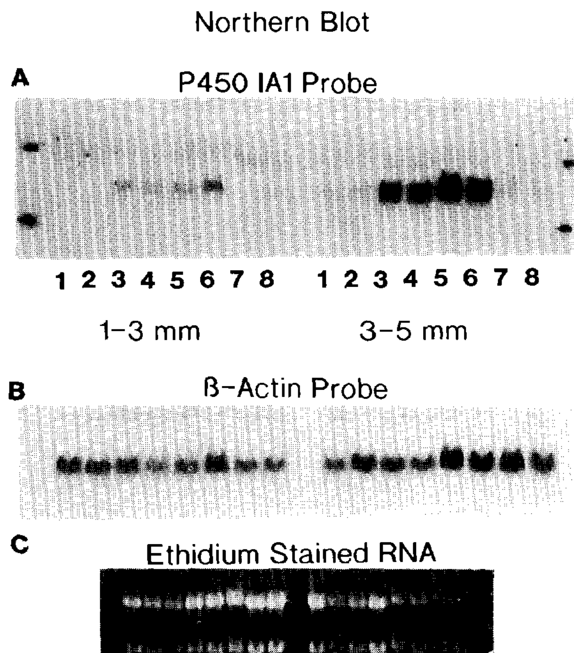

Fig. 3. Effect of 3MC and dexamethasone on CYP 1A1 expression in primary granulosa cells. Cells were plated at an original density of 5×10^6 cells/60 mm dish and incubated in growth medium for 5 days, with a medium change at day 3. On day 5 the cells were washed with PBS and incubated for 18 h in DMEM alone (lanes 1–2), 5 μM 3MC (lanes 3–4), 5 μM 3MC and 1 μM dexamethasone (lanes 5–6), or 1 μM dexamethasone (lanes 7–8). Ten μg of RNA was fractionated on agarose/formaldehyde gels, blotted to nylon membrane, and probed with the indicated cDNAs. Autoradiography was for 3 days for CYP 1A1 and 3 h for β-actin.

RESULTS

Our long term goal is to identify and characterize the E_2 hydroxylases expressed in ovarian tissue. To initiate our studies, we investigated the expression of mRNAs for known hepatic $P450$ E_2 hydroxylases [3–5]. Two ovarian cell culture systems were tested; the MDG2.1 cell line and primary porcine granulosa cells. MDG2.1 cells offer the convenience of immortalized cell lines while retaining some characteristics of the primary cell population; thus, they are a useful model system to study granulosa cell function [9]. For comparative purposes, primary cultures of porcine granulosa cells at two levels of differentiation were also examined. Both cell populations were treated with inducers of $P450$s with E_2 hydroxylase activity, the RNA isolated, and probed with cDNAs for the different $P450$s. $P450$s tested were CYP 1A1 and 1A2 (cells treated with 5 μM 3-methylcholanthrene), CYP 3A3 (cells treated with 10 μM pregnenolone-16α-carbonitrile), and CYP 3A4 (cells treated with 100 ng/ml rifampicin). Initial studies demonstrated that of the $P450$ E_2 hydroxylases examined, only CYP 1A1 was expressed and inducible in both primary granulosa cells and MDG2.1 cells. Subsequent work focused on this RNA.

Time course studies revealed that CYP 1A1 RNA was rapidly induced in MDG2.1 cells, reaching maximum levels within 2 h after 3MC administration (Fig. 1). The expression of CYP 1A1 RNA could be completely inhibited in MDG2.1 cells by a 4 h co-incubation with 5 μg/ml actinomycin D and 5 μM 3MC (Fig. 2, lanes 5–6). Based on the results presented in Fig. 1, this 4 h incubation with 3MC was sufficient for complete induction of CYP 1A1 RNA. Surprisingly, expression of CYP 1A1 RNA was observed in the control (i.e. non-3MC treated) MDG2.1 cells (Fig. 2, lanes 1–2). Although not seen in the first experiment (Fig. 1, lanes 1–2), expression of CYP 1A1 RNA in MDG2.1 cells was observed in control samples from the MDG2.1 cells in 3 of 5 different Northern blots with different batches of cells.

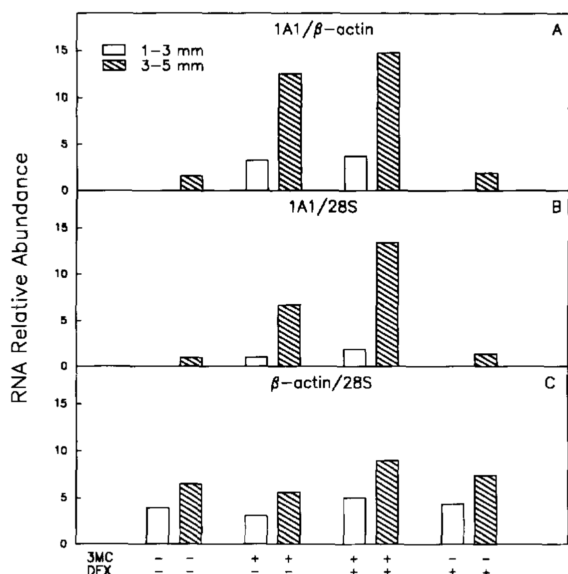

Fig. 4. Densitometric analysis of Northern blot in Fig. 3. CYP 1A1 and 28 S ribosomal RNAs were quantitated by laser densitometry and β-actin was quantitated with a Betagen Scanner. Values presented are mean values ($n = 2$).

Dexamethasone treatment (1 μM for 18 h) was without effect on CYP 1A1 RNA levels in MDG2.1 cells with or without 3MC (data not shown). However, dexamethasone enhanced the 3MC induction of CYP 1A1 RNA in primary granulosa cells [Fig. 3(A), lanes 5–6 vs lanes 3–4]. This effect of dexamethasone in primary granulosa cells is most obvious in experiments with granulosa cells isolated from 3–5 mm follicles. The effect of dexamethasone co-induction on CYP 1A1 RNA is less pronounced when CYP 1A1 RNA levels are normalized to β-actin [Fig. 4(A), 3MC – / + DEX]. It appeared from the Northern blot of granulosa cell RNA that β-actin RNA is increased by dexamethasone [Fig. 3(B), lanes 5–8], particularly in RNA from 3–5 mm follicles. As an alternative method for normalization, a photographic negative of the ethidium bromide stained 28 S ribosomal RNA [Fig. 3(C)] was scanned by laser densitometry. The 28 S ribosomal RNA was used to normalize both CYP 1A1 [Fig. 3(A)] and β-actin [Fig. 3(B)] RNAs. These ratios are shown graphically in Fig. 4(B) and 4(C). Dexamethasone increased the relative abundance of CYP 1A1 RNA 2-fold in 3MC-treated granulosa cells isolated from both 1–3 and 3–5 mm follicles [Fig. 4(B)]. Dexamethasone also had a slight effect on the relative abundance of CYP 1A1 RNA in non-3MC-treated granulosa cells from 3–5 mm follicles [a 38% increase, far left and right vertical hatched bars in Fig. 4(B)].

The β-actin/28 S ribosomal RNA ratio was also increased in cells treated with dexamethasone as compared to control cells [Fig. 4(C)]. In 3MC-treated cells dexamethasone increased the relative abundance of β-actin RNA by 63 and 60% (1–3 and 3–5 mm follicles, respectively) relative to non-dexamethasone treated cells. In the absence of 3MC, an increase in the relative abundance of β-actin RNA of 11 and 12% (1–3 and 3–5 mm follicles, respectively) was observed in dexamethasone-treated as compared to non-dexamethasone treated cells.

To examine hormonal and physiological influences on CYP 1A1 RNA expression, we incubated either MDG2.1 or primary granulosa cells with intracellular effectors or regulatory hormones. As shown in Fig. 5, an 18 h preincubation of 10 μM TPA followed by 4 h co-incubation with 5 μM 3MC completely inhibited CYP 1A1 RNA expression. One μM TPA was only partially effective as an inhibitor of CYP 1A1 expression, decreasing relative expression 43% compared to 3MC treated cells [Fig. 5 (bottom)]. Longer time periods (48–72 h) at a lower dose (100 nM) of TPA were not effective at inhibiting CYP 1A1 RNA expression. Other possible effectors of gene activation were also examined. Ten μM forskolin, 100 ng/ml follicle stimulating hormone, and 1% DMSO were added to the cell culture medium. These agents were without effect on CYP 1A1 RNA levels in either 3MC

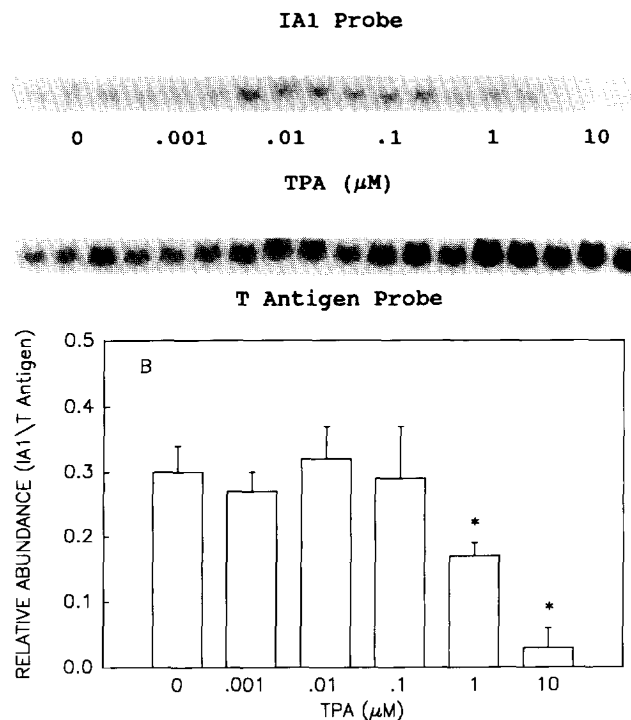

Fig. 5. Effect of TPA on CYP 1A1 RNA expression in MDG2.1 cells. (Top) Cells were plated in triplicate and the RNA analyzed by Northern blots as described in Fig. 1. Cells were preincubated with TPA at the concentration indicated for 18 h in DMEM/F12 medium, followed by a 4 h co-incubation with TPA and 3MC. Autoradiography was 48 h for CYP 1A1 and 18 h for the T antigen cDNA probe. (Bottom) Densitometric analysis of blots shown in (A). Values presented are the mean ± SD ($n = 3$). Asterisks represent values significantly different than control ($P < 0.01$). Values were determined by laser densitometry for CYP 1A1 and Betagen scanning for the T antigen.

treated or uninduced MDG2.1 cells, or in primary granulosa cells from developing (3–5 mm) follicles (data not shown).

To examine whether CYP 1A1 is expressed in non-cultured ovarian cells, we blotted total RNA isolated from ovarian follicles obtained after pigs had been treated with altrenogest for 30 days to synchronize the ovarian cycle. In this model estrus and the preovulatory surge occur between the evening of day 5 or the morning of day 6 [14]. RNA from five individual follicles obtained at 1, 3, 5, and 7 days (20 follicles total) after altrenogest withdrawal were probed with the CYP 1A1 cDNA. As can be seen in Fig. 6, 2 preparations of RNA were found to contain CYP 1A1 RNA, one each on days 5 and 7 after altrenogest withdrawal. Smaller and earlier follicles were negative (data not shown). The results suggest significant induction of CYP 1A1 in ovarian cell differentiation *in vivo*.

DISCUSSION

The results reported here demonstrate that CYP 1A1 is a strong candidate for the estrogen hydroxylase activity previously reported in ovarian cells [1, 15]. This enzyme is usually present in cells exposed to various polycyclic hydrocarbons, ligands for the Ah receptor. In addition, a ligand-independent (i.e. non-3MC treated controls) expression of CYP 1A1 RNA was observed in primary and immortalized granulosa cultured cells and in non-cultured cells.

It is unlikely that the ligand-independent expression of CYP 1A1 is a culture artifact for three reasons. First, expression was not detected in cells isolated from immature follicles. Second, ligand-independent expression was also observed in granulosa cells from developing follicles isolated from different batches of ovaries (3 of 4 different experiments) and cultured with different lots of media and sera. Third, the results are consistent with observations of other systems. For

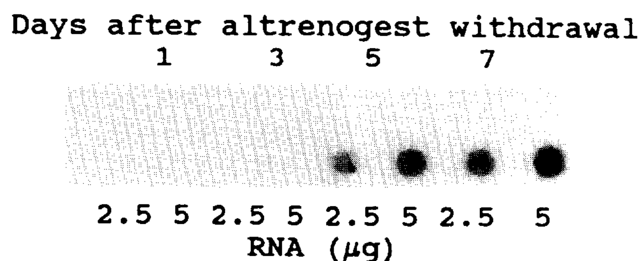

Fig. 6. Dot blot of RNA from non-cultured pig follicles. RNA was isolated as described in Material and Methods and applied to nylon membranes in the quantity indicated. RNA from 5 different follicles per withdrawal period (20 total) were probed with the CYP 1A1 cDNA probe. Data shown conform to two doses of RNA from the largest follicle in each of 4 ovaries (one from each day; 3–5 mm diameter on days 1 and 3; 7–10 mm on days 5 and 7). RNA from smaller follicles on each of these days failed to hybridize with the probe under these circumstances. Autoradiography was for 48 h.

example, ligand-independent induction of CYP 1A1 RNA has been reported in rabbit liver [16] and F9 embryonal carcinoma cells [17]. These observations, while circumstantial, are consistent with a role for CYP 1A1 in ovarian E_2 hydroxylation *in vivo* and in normal ovarian physiology.

Hepatic CYP 1A1 RNA levels are increased by co-treatment with glucocorticoids [18, 19]. In conjunction with a primary inducer (e.g. 1,2-benzanthracene), dexamethasone increases hepatic CYP 1A1 RNA levels 2-fold (relative to 28 S ribosomal RNA) when compared to the effect of the primary inducer alone. A similar level of co-induction was observed in primary ovarian granulosa cells from both 1–3 and 3–5 mm follicles. A dexamethasone effect on CYP 1A1 RNA levels was not observed in the immortalized MDG2.1 cell line, probably because of a loss of the glucocorticoid receptor or some other trans acting DNA transcription factor. Hepatic induction is mediated through transcriptional activation of the CYP 1A1 gene, with binding of the glucocorticoid receptor localized within the first intron [18]. These results indicate that hepatic and ovarian granulosa cell induction of CYP 1A1 share some common regulatory features.

In our experiments β-actin RNA appeared to be induced by dexamethasone. The ratio of β-actin/28 S ribosomal RNA is greatest in all RNA samples from dexamethasone-treated granulosa cells when compared to the non-dexamethasone-treated sample. Dexamethasone induction of β-actin RNA has been reported previously [20] but the mechanism of action is unknown. These data indicate that β-actin RNA is not useful for normalizing RNA in dexamethasone-treated granulosa cell populations.

We were interested in determining whether co-ordinate regulation of ovarian sterol synthesis and metabolism exists in granulosa cells. Gonadotropin stimulation of granulosa cells initiates a cascade of events, including steroidogenesis and cell growth, through cAMP activation of protein kinase A (PKA) [21]. An increase in E_2 hydroxylase activity in ovarian cells was also reported after injections of gonadotropins in rats [15]. Incubating MDG2.1 and primary granulosa cells with chemical and hormonal stimulators of PKA (FSH, cAMP, and forskolin) showed that these agents were without effect on CYP 1A1 RNA levels. In contrast, a regulator of protein kinase C (TPA) could completely inhibit the 3MC-induced expression of CYP 1A1 RNA in MDG2.1 cells. Other studies have shown that phorbal esters either stimulate or inhibit CYP 1A1 RNA expression through the protein kinase C dependent pathway in a biphasic and temporal manner [22]. The inhibition of CYP 1A1 gene expression may occur through inhibition of Ah receptor phosphorylation, thus preventing transcriptional activation of the CYP 1A1 gene [23, 24].

Although CYP 1A1 levels do not appear inducible by pKA stimulators, our preliminary studies indicate that

levels of this mRNA were increased during follicular growth *in vivo*. While more detailed studies will be required to finalize this assessment, these results parallel our previous observations indicating induction of estrogen-2-hydroxylase activity in comparable preovulatory porcine follicles [1]. Several explanations are possible for these results. First, it is possible that FSH may act in an indirect manner to increase enzymatic activity, for example, through peptide or steroid autocrine/paracrine mechanisms involved in a general regulation of cell growth. It is interesting to note that the PKC enzymes are important regulators of cell growth [25] and, through induction of metabolic enzymes such as CYP 1A1, may regulate concentrations of intracellular ligands (estrogens) which affect growth. This possibility has been discussed previously [26]. A second explanation is that the gonadotropin-dependent component of E_2 hydroxylase activity (corresponding to CYP 1A1 RNA levels) may have been maximally stimulated by gonadotropins during follicular development *in vivo* and serum containing culture. In this case, the capability of certain xenobiotics to increase RNA levels over those found by endogenous stimulation is not surprising. Alternatively, a second, as yet unidentified, gonadotropin-regulatable and tissue specific E_2 hydroxylase may exist in granulosa cells.

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