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Regulation of Cytosolic Phospholipase A_2 in Rat Endometrial Stromal Cells: The Role of Epidermal Growth Factor

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ABSTRACT The effect of epidermal growth factor on the levels of cytosolic phospholipase A_2 mRNA and protein in cultured rat endometrial stromal cells isolated from uteri sensitized for the decidual cell reaction was examined. Treatment with epidermal growth factor increased the steady-state cytosolic phospholipase A_2 mRNA and protein levels as demonstrated by Northern and Western blot analyses, respectively. Immunocytochemical analysis demonstrated an increase of cytosolic phospholipase A_2 protein in most cells, as opposed to a small subpopulation of cells in culture. These results show that epidermal growth factor causes an increase in steady-state cytosolic phospholipase A_2 mRNA and protein levels in rat endometrial stromal cells from uteri sensitized for the decidual cell reaction. Epidermal growth factor receptor ligands may regulate cytosolic phospholipase A_2 and thus prostaglandin production in the endometrial stromal cells during implantation. *Mol. Reprod. Dev. 52:335-340,* 1999.

Key Words: epidermal growth factor; cytosolic phospholipase A_2; endometrial stromal cells; uterus

INTRODUCTION

The mobilization of arachidonic acid (AA) from membrane phospholipids is believed to be a rate-limiting step in the production of eicosanoids (Pruzanski and Vadas, 1991). A group of enzymes, called phospholipase A_2 (PLA_2), is able to mobilize AA from the sn-2 position of membrane phospholipids (Flower and Blackwell, 1976; Irvine, 1982). At present, there are five well-described mammalian calcium-dependant PLA_2 enzymes. These include four **-14** kDa secretory PLA_2s ($sPLA_2$) and an 85 kDa cytosolic PLA_2 ($cPLA_2$) (reviewed by Clark et al., 1995; Leslie, 1997; Tischfield, 1997). The $sPLA_2$s share homology to each other whereas $cPLA_2$ shares no homology with other known PLA_2 enzymes. Further, $cPLA_2$ is the only well characterized PLA_2 enzyme that preferentially hydrolyzes sn-2 AA. Female mice deficient in $cPLA_2$ ("knock-out") become pregnant less frequently and have a significantly decreased litter size that might be due to a defect in blastocyst implantation (Bonventre et al., 1997) related to the requirement for prostaglandin (PG) synthesis for implantation (Kennedy, 1990; Smith, 1991). Although it might have a role in implantation by regulating PG synthesis, nothing is known about $cPLA_2$ gene expression in the uterus during implantation.

Previously, it has been shown that epidermal growth factor (EGF) modulates PG production in cultured endometrial stromal cells, increasing PG production in cells isolated from mice, human, and pig uteri (Ishihara et al., 1990; Paria et al., 1991; Zhang et al., 1992). We have previously found that EGF causes transcription- and translation-dependent increases in prostaglandin (PG) production in rat endometrial stromal cells isolated from rat uteri sensitized for decidualization (Bany and Kennedy, 1995). Although EGF increased the expression of cyclooxygenase-1 and -2 (Bany and Kennedy, 1997), cyclooxygenase activity was not the rate-limiting step for prostaglandin E_2 production in these cells. Thus, since the rate-limiting step in PG production is presumably the mobilization of AA from cellular stores, the purpose of the present study was to determine if these rat endometrial stromal cells express $cPLA_2$ mRNA and protein and whether EGF regulates their levels.

MATERIALS AND METHODS

Animals

Female Harlan Sprague-Dawley rats (200-225 g) (Harlan Sprague-Dawley Inc., Indianapolis, IN) were housed under temperature- and light-controlled conditions (lights on from 0500 to 1900 hr) with free access to food and water. The animals were ovariectomized under diethyl ether (BDH Inc., Toronto, Ontario, Canada) anesthesia and allowed at least 5 days to recover. To obtain rats with uteri sensitized for decidualization, estradiol and progesterone in sesame oil (Sigma Chemical Company, St. Louis, MO) were administered subcutaneously as described elsewhere (Bany and Kennedy, 1995).

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Endometrial Stromal Cell Isolation

Endometrial stromal cells were obtained from the sensitized uteri and plated in 24-well plates (Becton-Dickson, Lincoln Park, NJ) either with or without plastic Thermanox" coverslips (Nunc Inc., Naperville, IL) as described elsewhere (Bany and Kennedy, 1995, 1997). The resulting cultures of attached cells were free of contaminating epithelial-type cells as indicated by the absence of positively-staining cells for cytokeratin by immunocytochemistry (data not shown).

After incubation in serum-free medium (DMEM:Fl2 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 1.25 µg/ml fungizone; Gibco BRL, Burlington, Ontario, Canada) for 48 hr, the cells were incubated with or without 40 ng/ml EGF (Gibco BRL) for 24 hr in serum-free medium. Under control conditions, the cells undergo decidualization as indicated by increased alkaline phosphatase activity (Bany et al., 1998), expression of urokinase-type plasminogen activator (Zhang et al., 1996) and other components of the tissue remodeling cascade (Nuttall and Kennedy, 1998). The treatment with EGF has been previously determined to provide the best and most consistent stimulation of PG production (Bany and Kennedy, 1995). At the end of the 24 hr incubation period, the cells were washed with Dulbecco's phosphate buffered saline (Gibco BRL) and harvested.

Northern Blot Preparation and Hybridization

The cells were lysed into RNA isolation buffer (4 M guanidium thiocyanate, 5% ß-mercaptoethanol, 0.5% sodium lauryl sarcosinate, 25 mM sodium citrate pH 7, 0.1% antifoam A; Sigma). Total RNA was then isolated using a previously described method (Chomczynski and Sacchi, 1987). Northern blots containing samples of total RNA (10 µg/lane) were prepared as previously described (Bany and Kennedy, 1997). The membranes were prehybridized for 3 hr in buffer containing 1 M NaCl, 50 mM Tris, 2.2 mM sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS; Sigma), 10X Denhardt's reagent (Sambrook et al., 1989), pH 7.5 at 65°C for 2 hr. The cPLA$_2$ probe (20 ng) was prepared as described below and labeled by the random priming technique in the presence of ^{32}P-dCTP (Amersham) using Random Primer Kit (Gibco). Hybridization was carried out at 65°C for 18 hr followed by two washes with 2X SSC (0.03 M sodium citrate, 0.3 M NaCl, pH 7)-l% SDS for 5 min, one wash in 1X SSC-1% SDS at 65°C for 20 min and then 2 washes in 0.1 **x** SSC-0.1% SDS for 15 min. The membranes were then analyzed using phosphorimaging screens and a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Membranes were stripped of hybridized probe by immersion in 1 mM Tris, 1 mM EDTA, 0.1X Denhardt's reagent, pH 8, for 2 hr at 75°C. Next, they were probed with a cDNA for mouse 18S ribosomal RNA (rRNA) (generous gift from Dr. Dylan Edwards, University of East Anglia, Norwich), labeled as above, in order to determine the relative amounts of RNA loaded into each lane and transferred to the membranes. The relative level of cPLA$_2$ messenger RNA (mRNA) was determined by image analysis using ImageQuaNT Software (Molecular Dynamics) and the results were expressed as the ratio of cPLA$_2$ mRNA signals to 18S rRNA signals.

cDNA of rat cPLA$_2$ was prepared by reverse transcription-polymerase chain reaction (RT-PCR). Rat kidney mRNA (2.5-5 µg total RNA) was reverse transcribed using Superscript Reverse Transcriptase (Gibco BRL) and the downstream PCR primer (discussed later in this article) by the manufacturer's recommended method. One-tenth of the reverse transcribed cDNA was then subjected to PCR in a final volume of 50 µl. The PCR reaction contained lX reaction buffer, 0.2 mM dNTPs (dCTP, dGTP, dATP and dTTP), 1.5 mM $MgCl_2$, 0.1 µM of each primer (5'-GACGTGTCAGAGCTGATGTT-3'; 5'-GAGACAGTGGATACGATGTG-3') and 1 unit of Taq polymerase (Gibco BRL). The samples were subjected to 35 cycles of annealing (60°C, 1 min), extension (72°C, 2 min) and melting (94°C, 1 min) in a thermocycler (Perkin-Elmer Cetus Instruments). The resulting 508 base pair PCR product representing nucleotides 1181-1688 of the rat cPLA$_2$ gene was isolated using DEAE cellulose paper after 1.5% agarose gel electrophoresis as described previously (Sambrook et al., 1989).

Methods similar to that of Kovalic et al. (1991) were used to prepare a vector for directly cloning the cPLA$_2$ RT-PCR product. Briefly, partially complementary 36-mers were synthesized (5'-AATTCCCATGGATAACATGGCCAACAAAACCATGGG-3' and 5'-AATTCCCATGGTTTTGTTGGCCATGTTATCCATGGG-3'). They were annealed and ligated to an EcoRI-digested pBluescript plasmid (Stratagene, Mississauga, Ontario, Canada). This vector was then digested with XcmI (New England Biotech, Mississauga, Ontario, Canada) yielding a linearized vector with single unpaired terminal 3'-deoxythymidylate residues. The cPLA$_2$ RT-PCR product was then directly cloned into the XcmI site using T4 DNA ligase (Gibco BRL) as previously described (Sambrook et al., 1989). The cloned RT-PCR product was then sequenced for verification at the University of Calgary DNA Services (Calgary, Alberta, Canada).

Western Blots

Cells were washed with PBS, scraped into boiling lysis buffer (1% SDS, 10 mM Tris, pH 7.4) and transferred into 1.5 ml microfuge tubes (VWR-Canlab, Mississauga, Ontario, Canada). After placing the tubes in boiling water for 5 min, protein concentrations in the samples were determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Samples (50 µg) and prestained Rainbow Molecular Mass Markers (Amersham, Oakville, Ontario, Canada) were subjected to 10% SDS-polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970) and then transferred to Immobilon™-P membranes (Millipore Corporation, Bedford, MA) by electroblotting (Towbin et al., 1979). The mem-

branes were placed in blocking buffer (5% nonfat dry milk in TBS: 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) for 1 hr and then in blocking buffer containing affinity-purified anti-cPLA2 goat antibodies (0.2 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr. After six 5 min washes in TBS, the membranes were incubated with peroxidase-conjugated donkey anti-goat IgG (80 ng/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hr. After washing in TBS, cPLA2 was detected by enhanced chemiluminescence using an Amersham-ECL Kit (Amersham). Densitometry was carried out to determine changes in immunoreactive cPLA2 protein levels.

Immunocytochemistry

Cells grown on plastic coverslips were rinsed in phosphate buffered saline (PBS; 0.01 M phosphate, 0.9% NaCl, pH 7.4), fixed in ice-cold methanol (BDH) for 5 min, air dried, then rehydrated in PBS. To quench endogenous peroxidase activity, the cells were incubated in 0.1% hydrogen peroxide in PBS for 5 min. Cells were then blocked with PBS containing 5% normal donkey serum (PBS-NDS) for 30 min at room temperature followed by an overnight incubation at 4°C with affinity-purified goat antibodies against cPLA2 (0.1 µg/ml; Santa Cruz Biotechnology) in PBS-NDS. After three 10 min washes in PBS the cells were incubated with biotinylated donkey anti-goat IgG (1.8 µg/ml; Jackson ImmunoResearch Laboratories) in PBS-NDS for 1 hr, washed in PBS, then incubated with peroxidase-conjugated streptavidin (1 µg/ml; Jackson ImmunoResearch Laboratories) in PBS for **30** min. After three 5 min washes in water, immunoreactive cPLA2 in the cells was detected as a red color after incubation with 3-amino-9-ethyl-carbazole (AEC Substrate Kit, Dimension Laboratories Inc., Mississauga, Ontario, Canada). Special care was taken to ensure that all coverslips were incubated with the substrate for exactly 20 min followed by washing with PBS to stop the reaction. The cells were then counterstained with hematoxylin. To determine the specificity of the immuno-localization for each antibody, some coverslips were incubated with primary antibody preincubated for 2 hr at room temperature with a IO-fold excess by weight of control peptide (the peptide used to generate the antibodies).

Statistical Analysis

Analyses of variance were used to determine treatment effects. All statistical analyses were carried out using SAS statistical software (Cary, NC).

RESULTS

The effect of EGF on steady-state cPLA2 mRNA levels and immunoreactive protein levels in the cells were determined. As shown in Fig. 1A, cPLA2 mRNA was detected as a single ~3 kB band in samples from cells treated without or with EGF. As determined by image analysis of three independent samples from three separate cultures, treatment with EGF (40 ng/ml) for 24 hr significantly ***(P < 0.02)*** increased the cPLA2 mRNA:18S ratio (Fig. 1B). As shown in Fig. 2A, a protein of approximately 85 kDa was detected in both vehicle- and EGF-treated cells by Western blot analysis. As determined by densitometry, EGF caused a significant ***(P < 0.004)*** increase in the intensity of the signal for cPLA2 protein compared to vehicle-treated cells (Fig. 2B). When the anti-cPLA2 antibodies were preincubated with control peptide, no signal was detected on the Western blots. Since EGF caused an increase in cPLA2 protein in the cells, the cells were subjected to cPLA2 immunocytochemistry. As shown in Fig. 3A, cPLA2 immunostaining was barely detectable in cells treated with vehicle. The intensity of this staining was greater in the cells treated with EGF (Fig. 3B). When the anti-cPLA2 antibody was preincubated with control peptide, no immunostaining was detected (Fig. 3C).

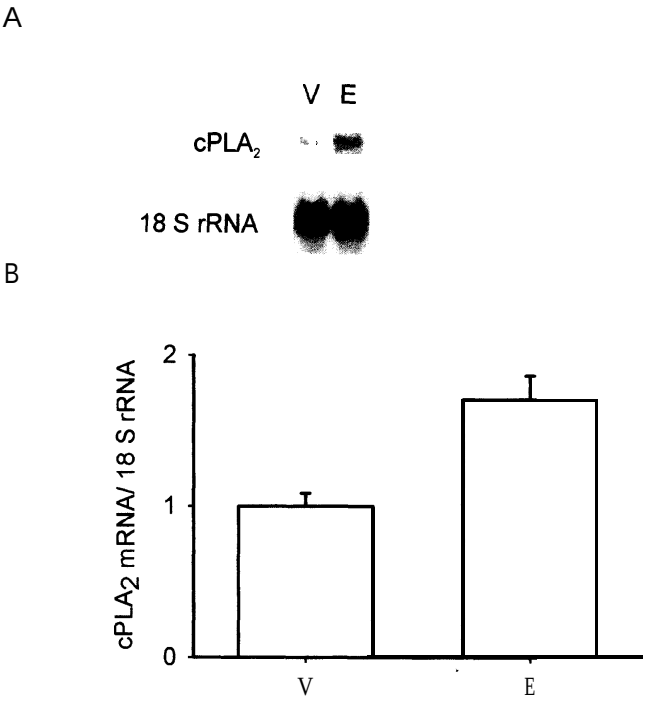

Fig. 1. Northern analysis of cytosolic phospholipase A2 (cPLA2) mRNA from rat endometrial stromal cells cultured with vehicle (V) or EGF (E; 40 ng/ml) for 24 hr. **(A)** Results from one representative experiment. **(B)** Mean (±SEM, N = 3) ratio of mRNA/18S rRNA signals, as determined by image analysis with the ratios for vehicle-treated cells set to 1.

DISCUSSION

The present study shows that EGF increases steady-state mRNA and immunoreactive protein levels of cPLA2 in cultured rat endometrial cells isolated from uteri sensitized for decidualization. The increase in steady-state cPLA2 mRNA levels in response to EGF may have been due to increased transcription and/or transcript stability. The increase in cPLA2 protein appeared to occur in most cells as determined by immunocytohemistry. Therefore the responses to EGF are likely to have occurred in most of the cultured cells

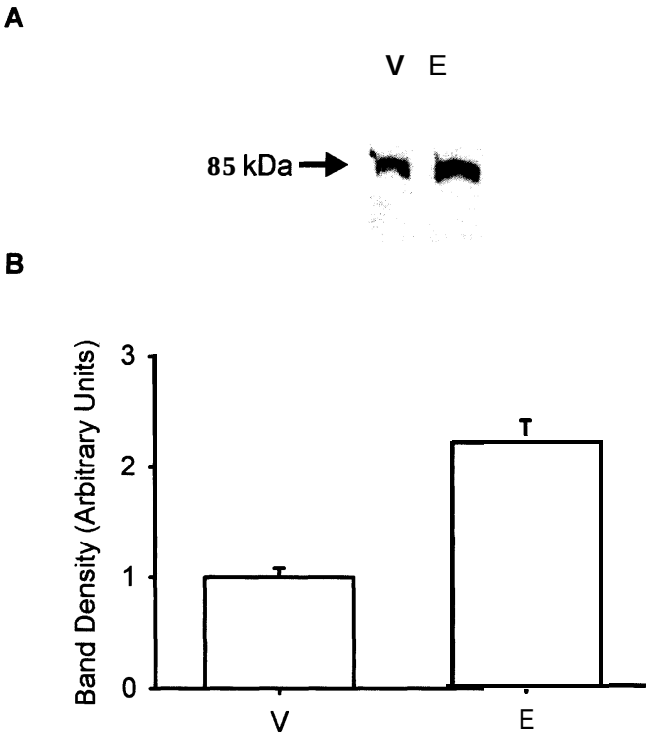

Fig. 2. Western analysis of cytosolic phospholipase A_2 ($cPLA_2$) proteins in rat endometrial stromal cells cultured with vehicle (V) or EGF (E; 40 ng/ml) for 24 hr. **(A)** Results from one representative experiment (50 μg total protein/lane). **(B)** Mean (± SEM, N = 3) density of immunoreactive protein signals, as determined by densitometry.

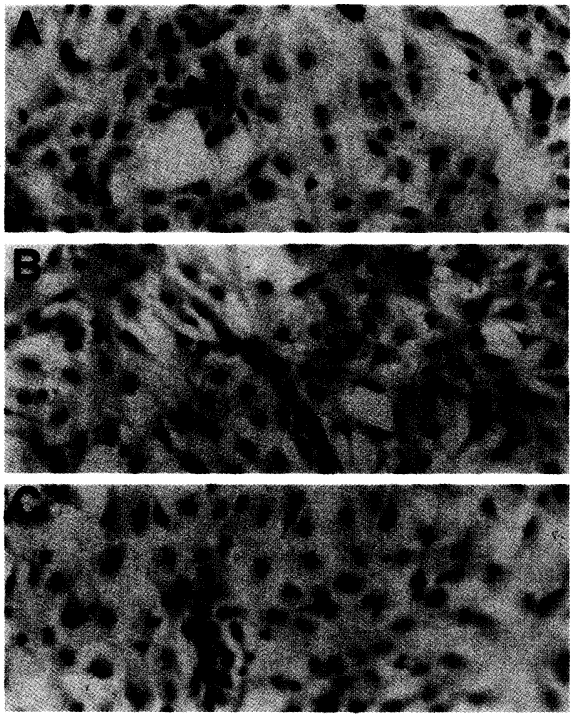

Fig. 3. Photomicrographs of rat endometrial stromal cells immunocytochemically stained with an antibody against cytosolic phospholipase A_2 ($cPLA_2$). Cells were cultured with vehicle **(A)** or EGF **(B;** 40 ng/ml) for 24 hr. Preincubation with control peptide (C) resulted in loss of staining. Magnification = 400 X.

as opposed to a small subpopulation of cells. Previous work has shown that EGF increases PG production, COX activity (Bany and Kennedy, 1995), and COX gene expression (Bany and Kennedy, 1997) in these rat endometrial stromal cells. The results of the present study extend these findings, and suggest that EGFR ligands may increase $cPLA_2$ gene expression in these cells. Notably, EGF is known to increase the synthesis of $cPLA_2$ in many cell types such as mesangial cells (Maxwell et al., 1993) and embryonic fibroblasts (Chepenik et al., 1994). In embryonic fibroblasts there is a coordinated up-regulation of both $cPLA_2$ and COX expression in response to EGF.

Several observations suggest that ligands of EGFR may be involved in modulating stromal cell function in the endometrium of rodents during implantation. First, any EGFR ligands that are present in the endometrium during implantation likely target primarily the stromal cells since EGFR protein and mRNA are present primarily in the endometrial stroma and not in epithelium during implantation in mice (Das et al., 1994a; Tong et al., 1996). Secondly, during the per&implantation period the rodent endometrium produces several EGFR ligands (Tamada et al., 1991; Das et al., 1994b, 1995; Paria et al., 1994) that may act in an autocrine or paracrine manner on stromal cells. If this is the case, the results of the present study support the notion that these EGFR ligands may modulate $cPLA_2$ gene expression in endometrial stromal cells during implantation.

PGs, particularly of the E-series, play an important role in blastocyst implantation and decidualization (reviewed by Kennedy, 1990; Smith, 1991). Inhibition of endometrial PG production prevents or delays implantation and decidualization in many species, including mouse (Lau et al., 1973; Saksena et al., 1976; Holmes and Gordashko, 1980), rat (Gavin et al., 1974; Garg and Chandhury, 1983; Wellstead et al., 1989), hamster (Evans and Kennedy, 1978), rabbit (El-Banna, 1980; Cao et al., 1985), pig (Kraeling et al., 1985), and ferret (Mead et al., 1988), effects which can be prevented by the administration of exogenous PGs (Lau et al., 1973; Saksena et al., 1976; Oettel et al., 1979; Holmes and Gordashko, 1980; Garg and Chandhury, 1983). Further, PG levels are higher in the endometrium at implantation sites compared to interimplantation sites in many species (Evans and Kennedy, 1978; Kennedy and Zamecnik, 1978; Sharma, 1979; Kennedy, 1983). Although essential for implantation, the source and control of PG biosynthesis in the endometrium during implantation are not known. From the results of the present study, it is tempting to speculate that that EGFR ligands may play a role in controlling $cPLA_2$ gene expression and thus PG production in endometrial stromal cells during implantation.

Although the regulation of $cPLA_2$ activity and gene expression has been studied in detail in other tissues,

there is no such information for the uterus. Total PLA_2 activity in the uterus appears to be under steroid hormone control (Dey et al., 1982; Pakrasi et al., 1983; Periwal et al., 1996). Further, general PLA_2 activity in the uterus changes during early pregnancy. In the rat uterus, general PLA_2 activity is low on days 3-4 of pregnancy (preimplantation) but increases on days 5 (day of implantation) and 6 (Cox et al., 1982; Novaro et al., 1996). Since PG levels increase in the endometrium of the rat at sites of implantation (Kennedy, 1977; Kennedy and Zamecnik, 1978; Pakrasi and Dey, 1982), it is tempting to speculate that the increase in total PLA_2 activity in the uterus occurs at implantation sites. The results of Moulton and Russell (1989) which show there is a reduction of AA-containing phospholipids at implantation compared to inter-implantation areas of the uterus on day 6 of pregnancy supports this speculation. Recent evidence suggests that $cPLA_2$ plays a key role in blastocyst implantation (Bonventre et al., 1997). However, to our knowledge, the localization and temporal changes in the expression of $cPLA_2$ and $sPLA_2$ genes in the uterus and embryo during the peri-implantation period has not been studied. Clearly, more work in this area is necessary.

CONCLUSION

The present study shows that EGF causes an increase in steady-state $cPLA_2$ mRNA levels in cultured endometrial stromal cells isol ated from rat uteri sensitized for decidualization. The increase in $cPLA_2$ mRNA levels were accompanied by increases in immunodetectable $cPLA_2$ protein in the cells. This study provides evidence that EGF induced increases in PG production by rat endometrial stromal cells may involve, at least in part, modulation of $cPLA_2$ gene expression.

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