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Effect of estradiol -17 β on arachidonic acid metabolism in sheep uterus: *in vitro* studies

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Abstract: *The effect of estradiol-17 β on Arachidonic acid (AA) metabolism in non-pregnant sheep uterus was studied under in vitro conditions. On incubation of uterine slices with estradiol-17β, the levels of prostaglandins were altered but not Lipoxygenase (LOX) products. Based on their analysis on conventional TLC technique, the Cyclooxygenase (COX) products $PGF_2\alpha$, 6-keto $PGF_1\alpha$ and PGE_2 were shown to be altered over an incubation period of 0 to 120 minutes. The LOX products, HPETEs and HETEs did not show any change upon incubation with estradiol-17β. This study gives a preliminary understanding of role of estradiol on AA metabolism.*
Keywords: *Lipoxygenase, Cyclooxygenase, estradiol-17β, Prostaglandins, HPETEs and HETEs.*

I. Introduction

The active metabolites of arachidonic acid, eicosanoids are grouped into cyclooxygenase products (prostaglandins [PGs] and thromboxanes) and lipoxygenase products (hydroperoxyeicosatetraenoic acids [HPETEs], hydroxyeicosatetraenoic acids [HETEs], leukotrienes [LTs] and lipoxins). Numerous studies suggest a role for the lipoxygenase and cyclooxygenase systems in reproduction [1,2,3]. Earlier we reported the abundant presence of 5-LOX, 12- & 15-dual LOX as well the COX enzyme in the non pregnant sheep uterus [4]. Estrogen is known to induce uterine proliferation, which depends on synthesis of the IGF1 growth factor. Both Igf1 regulation and uterine proliferation require the DNA binding function of the estrogen receptor (ER). Full ER function is needed to mediate other cellular signals of the growth factor for uterine growth [5]. Studies with estrogen receptor knockouts and estrogen depleted mice have shown that estrogen is essential for folliculogenesis and is necessary to maintain the female phenotype of ovarian somatic cells[6]. Estrogens also have a possible role in the development of ovary[7]. The role of LOX and COX enzymes was well established in the reproduction. Indomethacin, a potent inhibitor of COX, induced several ovulatory alterations, consisting of a decrease in the number of oocytes effectively ovulated, trapping of oocytes inside the luteinized follicle, as well as abnormal follicle rupture at the basolateral sides, with release of the oocyte and follicular fluid to the interstitium [8]. On the other hand, inhibition of the LOX pathway with the selective inhibitor NDGA, did not affect ovulation. The in vivo studies confirmed the importance of COX pathway in the ovarian development. The levels of various prostaglandins was shown to be decrease or completely suppressed by treatment of ovarian tissue with NDGA and indometahacin, potent COX inhibitors [9]. Recently it was shown that 5- and 12-LOX pathways modulate human myometrium contractility also[10]. Inhibition of cyclooxygenase and lipoxygenase pathways in combination was found to inhibit the estrogen and epidermal growth factor induced uterine growth, suggesting the involvement of eicosanoids in esradiol action. Chakraborty et al [11] showed that COX-1 mRNA increased in the mouse uterus when either estrogen or progesterone was given suggesting a possible role of estrogen on COX enzyme in the uterus. Even though the studies so far reported are emphasizing the roles of COX, LOX and estrogen on the uterine growth independently, there is no inter-relationship between the estradiol and eicosanoids in modulating their effects on the same system.

In order to test the hypothesis that the estradio-17 β exert its effect on the uterine growth and function by mediating through LOX and COX products, the study was designed to verify whether estradiol-17β could alter the product profile of LOX and COX. In vitro studies were done by incubating uterine slices with varying concentrations of estradiol-17 β for different intervals, and products of LOX and COX were analyzed on TLC.

II. Materials and Methods

Hematin, phenyl methyl sulphonyl fluoride (PMSF), diethyl dithio carbamate (DDC), Triton X-100 and N,N,N",N"-tetramethyl-p-phenylene diamine (TMPD) were purchased from Sigma Chemicals co., St. Louis, USA. DE-52 was from Whatman, PG standards and estradiol-17β are from Cayman Chemicals, Ann Arbor, USA. AA was from Nu Chek Prep, USA. All other chemicals were of analytical grade and from local chemical companies.

2.1. Tissue collection

Non pregnant sheep uteri were collected from a local slaughter house, transported on ice to the laboratory and immediately used. The tissues were processed free of fat tissue, ovaries and cervix.

Extraction of uterine lipoxygenase (LOX):The procedure described earlier was followed basically[4]. The non-pregnant sheep uterine tissue was minced and homogenized to 20% homogenate in 150 mM potassium phosphate, pH 7.4 buffer containing 1 mM ascorbic acid, 1 mM EDTA, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM KCl, 10 mM sodium metabilsufite, 1 mM PMSF and 250 mM sucrose. The homogenate was filtered through two layers of cheese cloth and centrifuged at 12,000 rpm for 20 min. The resulting supernatant was centrifuged at 33,000 rpm for 1 h and the supernatant (cytosol) was loaded on to DE-52 in batch-wise fashion and flowthrough was collected and checked for activity and protein. The matrix was washed with three volumes of homogenization buffer and then eluted with one volume of the same buffer containing 0.5 M NaCl and then checked for activity and protein. Proteins in the flow through were precipitated by subjecting to 100% ammonium sulfate $(NH_4)_2SO_4$ saturation. The precipitated proteins were subjected to activity assay. Protein concentrations were measured by Lowry's method [12].

2.2. Assay of LOX

Enzyme activity was measured spectrophotometrically by basically following the previously described method [13]. The reaction mixture contained 30-100 µg of uterine LOX in 150 mM citrate phosphate, pH 5.5 buffer at room temp. The reaction was initiated by addition of 250 µM AA and followed for 1 min at 235 nm for the formation of conjugated diene. Enzyme activity was defined as µmoles of hydroperoxides formed per min per ml enzyme.

2.3. Extraction, separation and identification of LOX products of AA

The separation of LOX products by thin layer chromatography was followed as described earlier[4]. The active enzyme was incubated with 250 µM AA in 150 mM citrate phosphate buffer, pH 5.5 for 2 min in 100 ml reactions. The reaction was terminated by acidifying the reaction mixture to pH 3.0 with 6 N HCl. The products (HPETEs/HETEs) were extracted twice with equal volumes of hexane: ether mixture. The organic phase was evaporated under rotary evaporator to total dryness. The residue was dissolved in ethanol.

The compounds were separated on TLC with mobile phase of diethyl ether: hexane: acetic acid (60:40:1) and identified based on co-chromatography with standards. The standards were generated in the laboratory using potato 5-LOX and green gram 15-LOX, following the protocol developed by Reddanna et al [14].

2.4. Extraction of uterine cyclooxygenase (COX)

The method described earlier was adopted [4]. Uterine tissue was homogenized in 100 mM Tris-HCl, pH 8.0 buffer containing 5 mM EDTA and 5 mM DDC. The homogenate was centrifuged at 12,000 rpm for 30 min at 4^0C. The supernatant was re-centrifuged at 33,000 rpm for 1 h in an ultracentrifuge. The microsomal pellet thus obtained was solubilized in 0.5% Triton X-100 for 30 min at 4^0C at slow stirring and the sample was again centrifuged at 42,000 rpm for 1 h and the resulting supernatant was used as the enzyme source.

2.5. Assay of COX

COX activity was measured spectrophotometrically by using a chromogenic assay based on the oxidation of TMPD during the reduction of PGG_2 to PGH_2. The assay mixture contained Tris-HCl buffer (100 mM, pH 8.0), hematin (1 µM), EDTA (5 mM) and COX enzyme source (100 µg). The reaction was initiated by the addition of 250 µM AA and 170 µM TMPD in the total volume of 1.0 ml. Change in absorbance was monitored over a period of 25 sec and the activity was calculated as the change in absorbance per min and specific activity as the change in absorbance per mg protein.

2.6. Extraction, separation and identification of COX products of AA

The uterine COX was incubated with 250 µM AA in 100 mM Tris-HCl, pH 8.0 buffer containing 5 mM EDTA, 1 µM hematin for 2 min at room temperature with constant stirring. The reaction was terminated by acidifying the mixture to pH 3.0 with 6 N HCl. The prostaglandins (PGs) were extracted twice with an equal volume of ethyl acetate : petroleum ether mixture (1:1 v/v). The organic phase was evaporated under dry N_2 gas to total dryness. The residue was dissolved in minimum volume of diethyl ether. The extracted PGs were separated on silica gel TLC plates using the solvent phase of water: saturated ethyl acetate : acetic acid : isooctane (51:80:25:80). The separated PGs were developed in iodine chamber and compared with the standards.

2.7. Incubation of estradiol-17β with uterine slices
Various methods were adopted to study different parameters.

2.8. Studies on COX

2.8.1. Time variation

Sheep uterine slices (5 gm) were incubated with 20 nM estradiol -17β for different time periods (0,30,60,120 min) in 100 mM Tris-HCl, pH 8.0 buffer containing 5 mM EDTA and 5 mM Diethyl dithio carbamate (DDC). At the end of incubation periods, the uterine slices were transferred into non-estradiol buffer of same composition. The slices were washed twice to make free of exogenous estradiol, homogenized in the same buffer and centrifuged at 12000 rpm for 30 min at 4^0C. The supernatant was re-centrifuged at 33000 rpm for 1 h in an ultracentrifuge at 4^0C. The microsomal pellet thus obtained was solubilized in 0.5% Triton X-100 for 30 min on ice at slow stirring. The sample was again centrifuged at 42,000 rpm for 1 h and the resulting supernatant was used as COX enzyme source.

2.8.2. Estradiol concentration variation

The study was followed as above except for the variation in the concentration of estradiol (0, 0.2, 20, 200 nM) and incubation period was maintained for 1 h.

2.9. Studies on LOX enzyme

2.9.1. Time variation

Sheep uterine slices were incubated with 20 nM estradiol-17 β in 100 mM Pot.Phos, pH 7.4 buffer containing 1 mM ascorbic acid, 1 mM EDTA, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM KCl, 10 mM sodium metabisulphate, 250 mM sucrose and 1 mM PMSF. After the respective incubation periods, the uterine slices were transferred into non-estradiol buffer of same composition, washed the slices twice and homogenized in the same buffer. The homogenate was centrifuged at high speed for 45 min. The resulting supernatant was used as LOX enzyme source.

2.9.2. Estradiol concentration variation

The study was followed as above except for the variation in the concentration of estradiol (0, 0.2, 20, 200 nM) and incubation period was maintained for 1 h.

2.10. Extraction of Products and analysis on TLC

The products of arachidonic acid substrate by both LOX and COX enzymes after estradiol treatment were extracted and analyzed in the same manner as described previously for non-estradiol treated uterine slices.

III. Results

3.1. Effect of estradiol on Cyclooxygenase enzyme system

The major prostaglandins formed by the sheep uterine microsomal COX were $PGF_2\alpha$, 6-keto $PGF_1\alpha$ and a modified PGE_2 [4]. There was an obvious response noted of the enzyme system towards the estradiol-17β. The activity of the enzyme was observed increasing as the incubation time was increased over the control (data not shown). The formation of products was also reflected the same (Fig.1 &2). $PGF_2\alpha$ formation was shown a clear increase at 30 min incubation period over the control and then rise was slowly reduced but higher than the control. PGE_2 formation was shown maximum at 60 min incubation period. 6-keto PGF1α formation was steadily increased up to 60 min incubation time and came steady thereafter (Fig.2). The observations made are showing a noted effect of estradiol 17-β on the production of COX products $PGF_{2\alpha}$, 6-Keto $PGF_{1\alpha}$ and PGE_2. The enhancement of the product formation is shifted from $PGF_{2\alpha}$ to PGE_2 to 6-Keto $PGF_{1\alpha}$ over the time course form 30 min to 120 min.

Studies were also conducted to understand the concentration of estradiol which could elicit the maximal hormonal effect, and observed that 20 nM estradiol is the optimum concentration at 60 min incubation time (Fig.3).

3.2. Effect of estradiol on lipoxygenase enzyme system

Similar studies were also conducted for the LOX enzyme and found that no significant effect of estradiol 17-β either on the activity or on the product formation of LOX (Fig.4).

IV. Figures

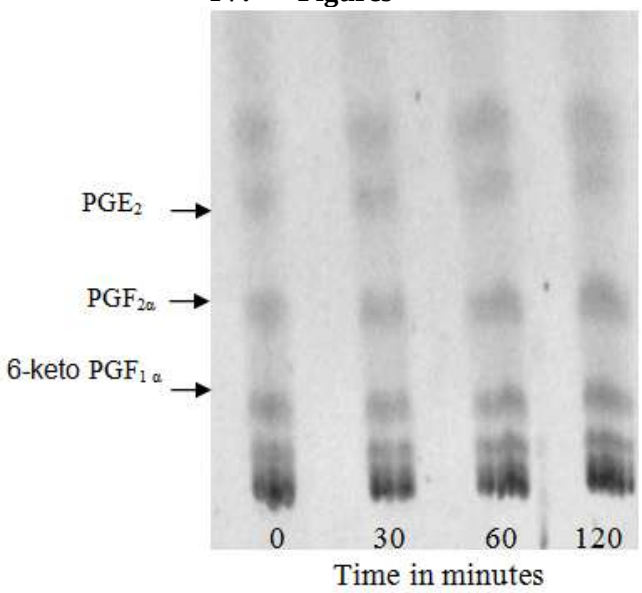


Figure 1. TLC analysis of effect of Estradiol-17β (20 nM) on COX products at various time periods [Sheep uterine slices were incubated with AA in 100 mM Tris-HCl, pH 8.0 buffer containing EDTA, hematin and 20 nM estradiol-17β for different incubation times. After the incubation time, the enzyme was extracted, incubated with AA and the products generated were extracted into ethyl acetate: petroleum ether mixture. The products were separated on silica gel TLC plates by using the solvent phase of water: saturated ethyl acetate: acetic acid: isooctane (51:80:25:80)]. Lane 1, PGs at 0 min incubation time; lane2, PGs at 30 min incubation time; lane 3, PGs at 60 min incubation time; lane 4, PGs at 120 min incubation time.

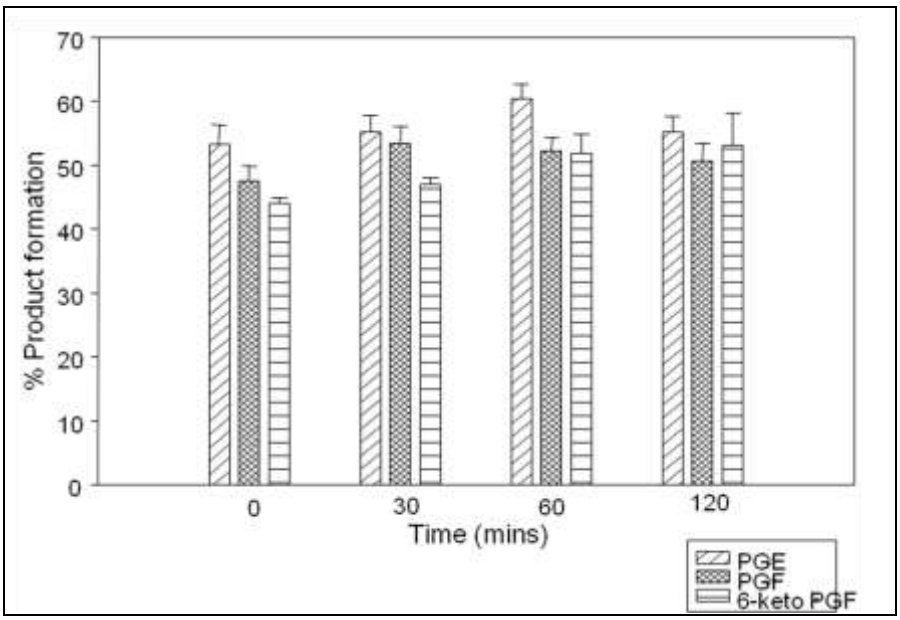
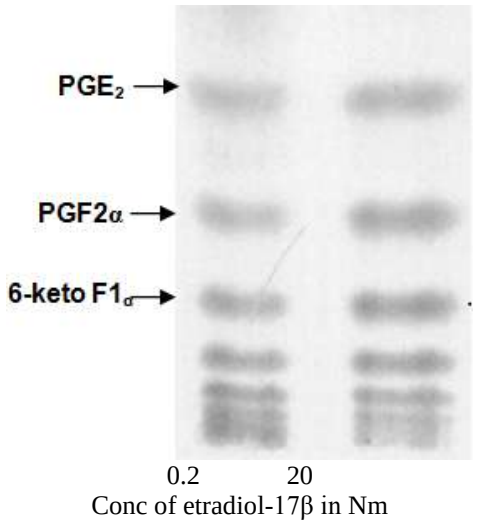


Figure 2. Graphical representation of fig.1



Figuer 3. TLC analysis of effect of estradiol-17β on COX products at various concentrations [Sheep uterine slices were incubated with AA in 100 mM Tris-HCl, pH 8.0 buffer containing EDTA, hematin and 20 nM estradiol-17β with different concentrations for 60 min incubation time. After the incubation time, the enzyme was extracted, incubated with AA and the products generated were extracted into ethyl acetate: petroleum ether mixture. The products were separated on silica gel TLC plates by using the solvent phase of water: saturated ethyl acetate: acetic acid: isooctane (51:80:25:80)]. Lane 1, PGs at 0.2 nM estradiol, lane 2, PGs at 20 nM estradiol.

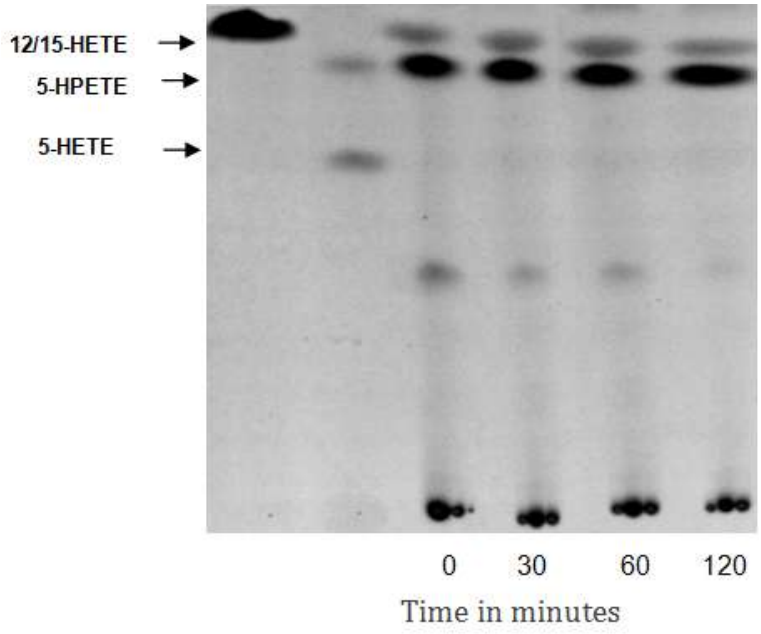


Figure 4. **T**LC analysis of effect of estradiol-17β (20 nM) on LOX products: [Sheep uterine slices were incubated with AA in 150 mM citrate phosphate buffer, pH 5.5 containing 20 nM Estradiol-17β for different time periods. The enzyme was extracted and the products generated were extracted into hexane:ether (1:1). The extracted products were separated on silica gel TLC by employing hexane:ether:acetic acid (40:60:1)]. Lane 1, standard 15-HETE; lane 2, Standard 5-HPETE and 5-HETE, lane 3, products generated at 0 min estradiol-17β incubation time; lane 4, products at 30 min incubation time; lane 5, products at 60 min incubation time; lane 6, products at 120 min incubation time.

V. Discussion

Eicosanoids, specifically prostaglandins E_2 and $F_2\alpha$ are known to play a key role in uterine functions. In the earlier study [4], it was reported that sheep uterine tissue metabolizes AA by two enzyme systems- LOX and COX. The LOX pathway includes 5-LOX which produces 5-HPETE and 12,15-dual LOX enzymes with resulting products of 12- and 15-HETE. The COX pathway leads to the formation of $PGF_2\alpha$, 6-keto-$PGF_1\alpha$ and PGE_2. The present study is focusing on the effect of female hormone estradiol 17-β on the production of HPETE, HETE and PGs. Analysis of the AA products was done on TLC which gave reproducible results. Estadiol-17β treatment affected the arachidonic acid metabolism via COX pathway in the uterine tissue by enhancing the production of prostaglandins. The maximum effect of estradiol was observed at 1 h incubation period and at 20 nM concentration. The LOX enzyme system had not shown any significant response to the treatment of estradiol even after 2 h incubation time period. This study forms the basis to show the mediation of estrogen action through arachidonic acid metabolism especially by COX pathway. The LOX pathway could not be altered by estradiol-17 β, giving an indication that LOX pathway could be regulated by other means in the uterine tissue. The observations made are of having physiological significance that during reproductive cycle, the alterations in the concentrations of estradiol-17β would reflect in the alteration of formation of products. These alteration of products especially PGs would have effect on proliferation, maturation and release of ovum. These results are also supported by the studies that COX-1 expression is specifically up-regulated in the uterine artery endothelium during high uterine blood flow states such as the follicular phase and, in particular, pregnancy [15]. Badavi and Archer [16] reported that COX expression was augmented by the administration of estrdiol in ovarectomized rat. The luteal phase is a time of relatively low E_2 whereas the follicular phase, in which the uterus is readied for implantation, is a time of higher E_2, COX expression was high during high serum estradiol level in turn increase in the PG production during follicular phase [17]. Estradiol administration stimulated COX pathway leading to increased PGF2 production in mammalian system [18]. Cox-1 protein, the key enzyme in the production of PGI_2 from arachidonate, exhibits a marked increase in expression during pregnancy and follicular phase [15].When both estrogen and progesterone are elevated in ovine pregnancy, the potent vasodilator prostacyclin (PGI_2) is also elevated in systemic arterial and uterine venous plasma [19]. Ospina et al., [20] found that 17-β estradiol increases rat cerebrovascular prostacyclin synthesis by elevating cyclooxygenase-1 and prostacyclin synthase. Our studies are suggesting an inter-relationship between AA metabolism and estrogen action in the reproductive tissue which may be extended further.

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