17-β-Estradiol upregulates COX-2 in the rat oviduct

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Abstract

We investigated the regulation of cyclooxygenase-2 (COX-2) by 17-β-estradiol (E_2) in the rat oviduct. We observed that COX-2 is expressed mainly in proestrous and estrous stages, periods under estrogenic influence. While exogenous administration of E_2 (1 μg/rat) significantly increased COX-2 protein levels, progesterone did not modify it. COX-2 was mainly localized on oviductal epithelial cells from estrogenized rat. Induction of COX-2 expression by E_2 was partially reverted by tamoxifen (1 mg/rat), an E_2 receptor antagonist. Estradiol treatment also increased prostaglandins (PGs) synthesis: 6-keto-$PGF_{1\alpha}$ (40%), a stable metabolite of prostacyclin (PGI_2), $PGF_{2\alpha}$ (40%) and PGE_2 (50%). Tamoxifen completely suppressed this enhancement. In order to discriminate which isoform of COX was implicated in the stimulatory effect of E_2 on PGs synthesis, oviducts were preincubated with meloxicam (Melo: 10^{-9} M) or NS-398 (10^{-7} M), two selective COX-2 inhibitors. Both Melo and NS-398 abolished the increase of PGs synthesis stimulated by E_2. All together, these data indicate that E_2 could upregulate COX-2 expression and activity in the rat oviduct and that the stimulatory effect of E_2 may be receptor-mediated.
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Keywords: Cyclooxygenase 2 (COX-2); Estradiol; Rat oviduct

1. Introduction

The oviduct plays an essential role during female reproduction by regulating the transport of gametes and embryos, and provides a specific microenvironment for fertilization and for primary stages of embryonic development [1]. Oviductal functions are dependent on regulation of smooth muscle contractility, ciliary beats of epithelial cells and synthesis of oviduct-derived factors. Several agents such as ovarian hormones [2], nitric oxide [3,4], prostaglandins (PGs) [5,6], cytokines [7] and endothelial growth factors [8] are known to contract or relax oviductal smooth muscle and to modulate ciliary beats.

Prostaglandins are involved in a variety of physiological processes including inflammation, vasoconstriction/dilatation and reproductive mechanisms. The oviduct synthesizes PGs and these autacoids evoke significantly

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contractile effects which in turn modulate oviductal functions [9,10]. It has been known that each PG produces different effects on tubal smooth muscle *in vitro* [11,12]. $PGF_{2\alpha}$ increases tubal muscle contractility while PGE_2 inhibits circular muscle contraction and it is capable of inhibiting or stimulating longitudinal muscle at low or high concentration, respectively [11].

Cyclooxygenase (COX) enzyme catalyzes the biosynthesis of PGH_2, a precursor for all PGs [13]. Two distinct COX isoforms have been identified. COX-1 is generally constitutive expressed and is reported to be critical for cytoprotective and homeostatic functions, whereas COX-2 is inducible by phorbol ester, mitogens, cytokines, serum [14–16] and is the major isozyme found in inflammatory and proliferating cell types [17]. Also, it is well known that steroid hormones regulate COX-2 synthesis in endothelial [18] and endometrial cells [19]. In addition, Arosh et al. [20], found that COX-2 from bovine endometrium can be regulated during the estrous cycle.

Recent studies demonstrated that human oviduct expresses both COX-1 and COX-2 isoforms. Also, the oviduct tissue has been postulated as the source and the target of prostacyclin [21]. These authors found that mouse oviduct also expresses both types of COX and that oviduct-derived PGI_2 may enhance embryo development in a paracrine fashion [22].

Prostaglandins could participate in different oviductal functions. There are evidences showing that PGs could regulate ovum transport in the rat oviduct [23].

On the other hand, it is well known that in mammals, egg transport through the oviduct is also regulated by ovarian steroids and by embryonic signals [24]. Orihuela et al. [25] described a correlation between increased oviductal protein synthesis and eggs transport acceleration in response to E_2.

It has been demonstrated that human chorionic gonadothropin (hCG) upregulates the expression of the COX-2 gene in mucosal cells from human fallopian tubes [26]. Despite the above data, there is still no evidence of estrogen regulation over COX-2 in the rat oviduct. Thus, we investigated the effect of 17-β-estradiol on COX-2 expression and activity in the rat oviduct.

2. Materials and methods

2.1. Drugs

17-β-estradiol, progesterone and tamoxifen were obtained from Sigma Chemical Co. (St. Louis, USA). [^{14}C]-arachidonic acid (specific activity 56 μCi/mmol was purchased from Amersham Corporation (Life Science, Bucckinghamshire, UK). Western-blotting reagents were obtained from Sigma Chemical and Biorad (CA, USA) Laboratories. Polyclonal COX-1 and COX-2 antibodies and NS-398 were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Meloxicam was purchased from Boehringer Ingelheim. The Prolong Antifade mounting medium was from Molecular Probes (Eugene, Oregon, USA). All other chemicals were analytical grade.

2.2. Animals

Wistar adult female rats (200–250 g body weight) were kept under controlled illumination (14 h light and 10 h dark) and temperature (24 °C). Pelleted food and water were supplied ad libitum. Vaginal cytology was monitored once a day and only animals showing a regular 4 day estrous cycle for more than two cycles were used. Rats were assigned at random to one of two groups: pregnant and cycling rats. To study the pattern of COX-2 expression in the estrous cycle, the oviducts were isolated from female rats in the different days of the cycle (metaestrous, diestrous, proestrous and estrous). Females assigned to pregnant group were caged overnight with fertile males. The following day was designated day 1 of pregnancy, if spermatozoa were present in the vaginal smear. To study the effect of steroids hormones on the expression and activity of COX-2, day 1 pregnant rats were treated with estrogen by injecting 1 μg of 17-β-estradiol in 0.2 ml of ethanol 30% i/p, or with progesterone by injecting 4 mg in 0.5 ml of vegetable oil s/c or with both 17-β-estradiol and progesterone [25,27]. To antagonize the effect of E_2, rats were treated with 1 mg of tamoxifen [28] in 0.2 ml of propilenglycol. Treated animals were killed by cervical dislocation 15 h after the injection. Control animals were treated with the vehicle solutions used to dissolve the steroid hormones and tamoxifen. Control and treated oviducts were collected, cleaned of fat and processed immediately or kept at −70 °C.

The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFYBO-CONICET) and carried out in accordance with the Declaration of Helsinki.

2.3. Western blot analysis

The expression of COX proteins was determined by Western blot analysis using a COX-1 and COX-2 specific antibodies. Oviducts were homogenized, sonicated for 10 s and centrifuged for 10 min at $2000 \times g$. Protein determination was assayed by the Bradford method [29], using bovine serum albumin as standard. Equal amount of proteins (100 μg/lane) were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose. Non-specific binding sites of the membrane were blocked using 5% dried non-fat milk in 0.05 M Tris-buffered saline (TBS) pH 7.2. Non-specifically bound antibody was removed by washing with TBS containing 0.1% Tween-20. Membranes were incubated with primary COX-1 (1:500) or COX-2 (1:1000) polyclonal antibodies (Cayman Chemical), followed by an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, 1:5000) as the secondary antibody and developed with 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP) and nitro blue tetrazolium (NBT). Rat seminal vesicles for COX-1 and macrophage lysates for COX-2, were used as positive controls. Protein bands were identified by molecular weight markers (BioRad). Signal intensity was quantified by measuring the densitometry of bands using Image J Software. COX-1 and COX-2 protein bands were normalized against β-actin.

2.4. Immunohistochemistry of cyclooxygenase-2 in the rat oviduct

Immunohistochemical localization of the enzyme COX-2 was performed on oviductal slices. Briefly, oviducts were frozen and cut by cryostat in sections of 5 μm thickness. Oviduct slices were fixed with 2% paraformaldehyde for 30 min at 4 °C. The sections were blocked with 5% BSA (Fraction-IgG free) and incubated overnight at 4 °C with primary polyclonal antibody against COX-2 (1:500; Cayman Chemical) and then incubated with goat Cy2-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch). The specificity of immunoreactivity was assessed by omitting the first antibody. Samples were then mounted in Fluoromount G mounting medium (Electron Microscopy Sciences). Immediately after preparation, tissue slices were examined under a confocal laser imaging system (Olympus FV1000, Tokyo, Japan).

2.5. PGs radioconversion: metabolism of $[^{14}C]$-arachidonic acid (AA)

Oviducts were placed in a Petri dish containing the modified Krebs-Ringer bicarbonate solution with glucose 11 mM (KRB). The metabolism of exogenous AA in the rat oviduct was determined by incubating the tissue for 60 min in KRB, containing 50 μCi/ml of $[^{14}C]$-AA at 37 °C, bubbled with a mixture of 95% O_2/5% CO_2 and constant shaking. Two oviducts were used for each determination.

To study if COX-2 was involved in PGs stimulation by E_2, the tissues were randomly pre-incubated with different COX-2 selective inhibitors for 60 min: meloxicam (10^{-9} M) or NS-398 (10^{-7} M) before adding $[^{14}C]$-AA. Controls were incubated in KRB medium alone. At the end of the incubation period, medium was acidified to pH 3 with HCl 1N in 1 volume of ethyl acetate and extracted twice for PGs. Pooled ethyl acetate extracts were dried under nitrogen. The residues were applied to silica gel TLC plates. Authentic prostanoids were run in parallel. Plates were developed in a solvent system of benzene:dioxane:glacial acetic acid (60:30:3 v/v). The position of the corresponding eicosanoids was visualized by spraying the dried plates with 10% phosphomolybdic acid. Radioactivity from the TLC zones for AA and for the different prostanoids was measured by liquid scintillation counting. Results were expressed as a percentage of the total radioactivity of the plates: % of cpm/100 mg ww (wet weight).

2.6. Statistics

Comparisons between values from different groups were performed using one-way ANOVA. Significance was determined using Tukey's multiple comparison test for unequal replicates. All values presented in this study represent means ± S.E.M. Differences between means were considered significant when *p* was 0.05 or less.
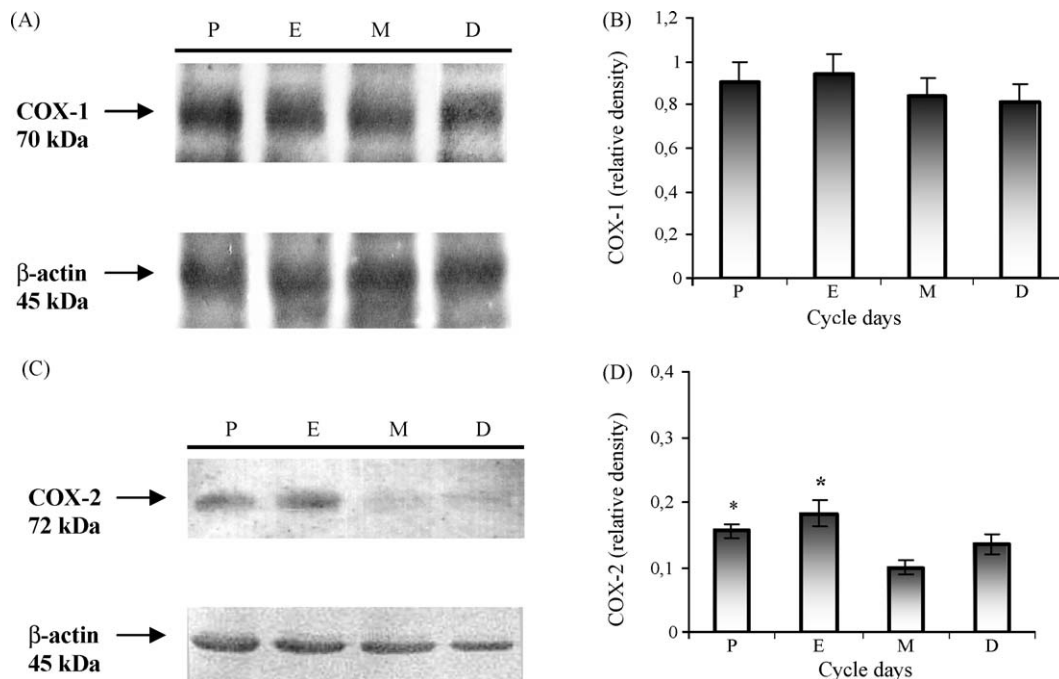
Fig. 1. Oviductal COX-1 and COX-2 expression in cycling rats. Oviductal proteins from proestrous (P), estrous (E), metaestrous (M) and diestrous (D) rats were loaded. A representative experiment is shown. (A) COX-1 representative western blot; (C) COX-2 representative western blot; (B) and (D) show quantified and normalized COX-1 and COX-2 protein levels to β-actin, respectively. Bars represent the mean ± S.E.M. of triplicate determinations from three representative animals. $^{*}p < 0.05$ vs. M.

3. Results

3.1. Expression of cyclooxygenase-2 in the rat oviduct

In order to determine whether the expression of oviductal COXs were modulated during the estrous cycle we analyzed, by western blot, both COX-1 and COX-2 protein levels in rat oviduct at different cycle days.

When we studied the expression of COX-1 protein in the oviduct, we observed that it was not modulated during the estrous cycle as shown in Fig. 1A and B.

Cyclooxygenase-2 was identified as a single band of 72 KDa (Fig. 1C). Densitometric analysis (Fig. 1D) revealed that even COX-2 expression was present in all cycle days, it was low during metaestrous and diestrous followed by a significant induction ($p < 0.05$) at proestrous and estrous.

As we observed that the expression of COX-2 was regulated during the estrous cycle and that its expression was correlated with plasma concentration of steroid hormones [30], we investigated if the treatment with estradiol and/or progesterone was able to modulate the protein levels of this enzyme in the rat oviduct. The administration of 17-β-estradiol produced a significant increase ($p < 0.05$) in COX-2 level (Fig. 2A and B lane 2). While progesterone did not modify COX-2 expression compared to the control band (lane 3), the administration of 17-β-estradiol together with progesterone (lane 4) augmented COX-2 expression to the same extent as 17-β-estradiol did alone.

Since treatment with E_2 resulted in a significant increase in COX-2 protein levels and progesterone did not modify them we decided to investigate the effect of this steroid in the regulation of COX-2 enzyme in the rat oviduct.

3.2. Effect of tamoxifen on oviductal COX-2 expression induced by E_2

To determine if the effect of E_2 on COX-2 induction was receptor specific, we treated the rats with 1 mg of tamoxifen (TAM), an antagonist of E_2 receptor. The results, shown in Fig. 3, indicate that TAM partially inhibited the effect of E_2 on COX-2 expression. Tamoxifen alone had not effect on the enzyme expression.
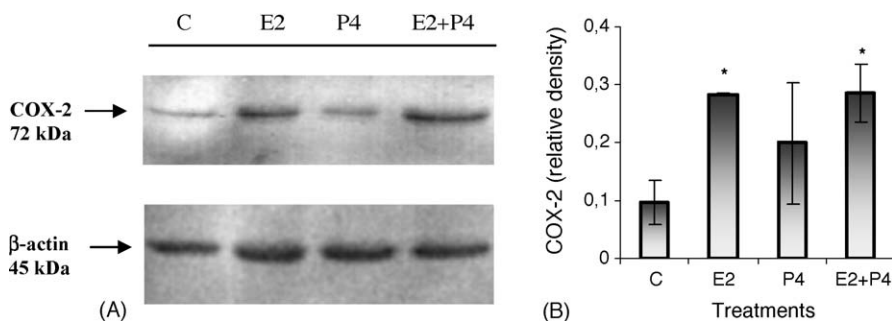
Fig. 2. Expression of COX-2 in the oviduct of rats treated with ovarian hormones. Day 1 pregnant rats were treated with vehicle (C: control), 17-β-estradiol (E$_2$: 1 μg/rat), progesterone (P$_4$: 4 mg/rat) or E$_2$ + P$_4$. Oviductal proteins from treated rats were loaded. Right panels show quantified and normalized COX-2 protein levels. Bars represent the mean ± S.E.M. of triplicate determinations from three representative animals. $^*p < 0.05$ vs. C.
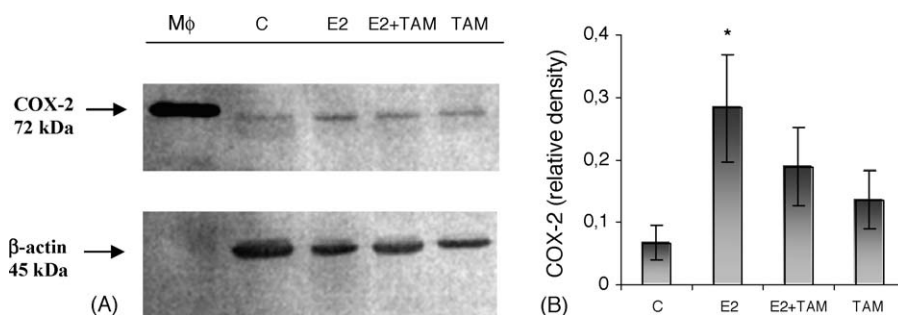

Fig. 3. Effect of tamoxifen on oviductal COX-2 expression induced by 17-β-estradiol. Day 1 pregnant rats were treated with vehicle (C: control), E$_2$ (1 μg/rat), Tamoxifen (TAM: 1 mg/rat) or E$_2$ + TAM. Oviductal proteins from treated rats were loaded. Right panels show quantified and normalized COX-2 protein levels. Bars represent the mean ± S.E.M. of triplicate determinations from three representative animals. $^*p < 0.05$ vs. C.

3.3. Immunochemical localization of oviductal COX-2

To study the localization of COX-2 in the oviductal tissue, immunohistochemistry was performed on oviductal slices using specific primary antibody. In the oviduct from estrogenized rats, COX-2 was localized in the luminal epithelium. As shown in Fig. 4C (close arrows), E$_2$ induced COX-2 expression in luminal epithelial cells, mainly in the perinuclear region and widespread in the cytoplasma. COX-2 expression was not observed in the smooth muscle cells (Fig. 4C; open arrow). In addition, weak stain was observed in the vascular endothelial cells (data not shown). Oviducts from control animals did not show stain when incubated with the antiCOX-2 antibody (Fig. 4A). Tamoxifen treatment partially inhibited the effect of the steroid, and TAM alone had no effect (Fig. 4 E and G, respectively).

Oviducts incubated without the primary antibody, did not show any unspecific stain.

3.4. Effect of 17-β-estradiol on oviductal prostaglandins (PGs) synthesis

We observed that the expression of COX-2 in the rat oviduct was regulated by E$_2$. Thus, we studied if E$_2$ was also able to modulate the synthesis of oviductal PGs, products of COX isoforms. As shown in Table 1, the conversion of arachidonic acid into its metabolites was significantly enhanced by 17-β-estradiol treatment (1 μg/rat): 6 keto-PGF$_{1\alpha}$

Table 1
Synthesis of PGs in rat oviduct treated with 17β-estradiol

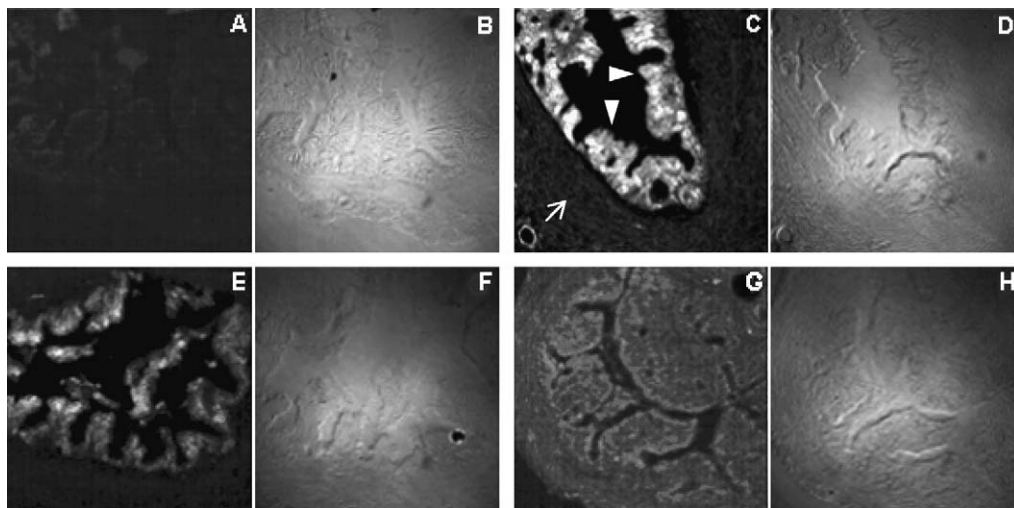| Treatment	6 keto-PGF$_{1\alpha}$	PGF$_{2\alpha}$	PGE$_2$	TXB$_2$
Control	6.29 ± 0.11	8.66 ± 0.15	8.59 ± 0.17	3.47 ± 0.08
E$_2$	8.90 ± 0.56*	11.80 ± 0.38*	13.04 ± 0.2*	3.66 ± 0.12

* $p < 0.001$ vs. control

Fig. 4. Immunolocalization of oviductal COX-2. Day 1 pregnant rats were treated with vehicle (C: control), E_2 (1 μg/rat), Tamoxifen (TAM: 1 mg/rat) or E_2 + TAM. Oviduct slices were incubated with primary polyclonal antibody against COX-2 (1:500). Negative control was performed omitting primary antibody. Luminal ephitelial cells expressed COX-2 in estrogenized rats (close arrows). Smooth muscle cells did not show stain (open arrow). Panel (A) oviduct from control females, (C) oviduct from estrogenized females, (E) oviduct from females treated with estradiol and tamoxifen, (G) oviduct from females treated with tamoxifen alone; (B), (D), (F) and (H) phase contrasts. Magnification: 40×.

(a stable metabolite of PGI_2; (40%)), $PGF_{2\alpha}$ (40%) and PGE_2 (50%). 17-β-estradiol had not effect on thromboxane B_2 levels.

In this case, TAM administration completely blocked ($p < 0.01$) PGs synthesis induced by E_2, even below control values (Fig. 5). Tamoxifen alone did not modify basal PGs synthesis. These results suggest that the effect of E_2 on COX activity could be receptor-mediated and that E_2 would not be involved in basal PGs production.

3.5. Participation of COX-2 in the 17-β-estradiol-induced PGs synthesis

In order to discriminate if COX-2 was implicated in the stimulatory effect elicited by 17-β-estradiol, we pre-incubated the oviductal tissues with meloxicam, a selective COX-2 inhibitor [31]. Meloxicam (10^{-9} M) completely
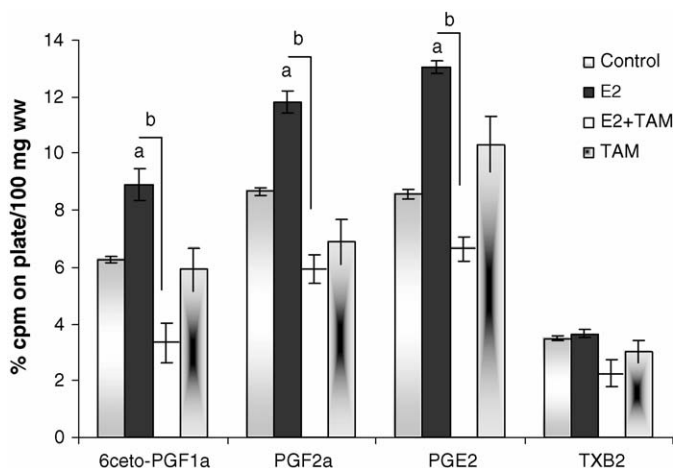

Fig. 5. Effect of tamoxifen on prostaglandins synthesis induced by 17-β-estradiol. Day 1 pregnant rats were treated with vehicle (C: control), E_2 (1 μg/rat), Tamoxifen (TAM: 1 mg/rat) or E_2 + TAM. The metabolism of exogenous AA was determined by incubating the oviducts for 60 min in KRB containing 50 μCi/ml of $[^{14}C]$-AA. Each bar represents the mean ± S.E.M. of triplicate determinations from four representative animals. Values are expressed as mean ± S.E.M. (a) $p < 0.01$ vs. C; (b) $p < 0.01$ vs. E_2.
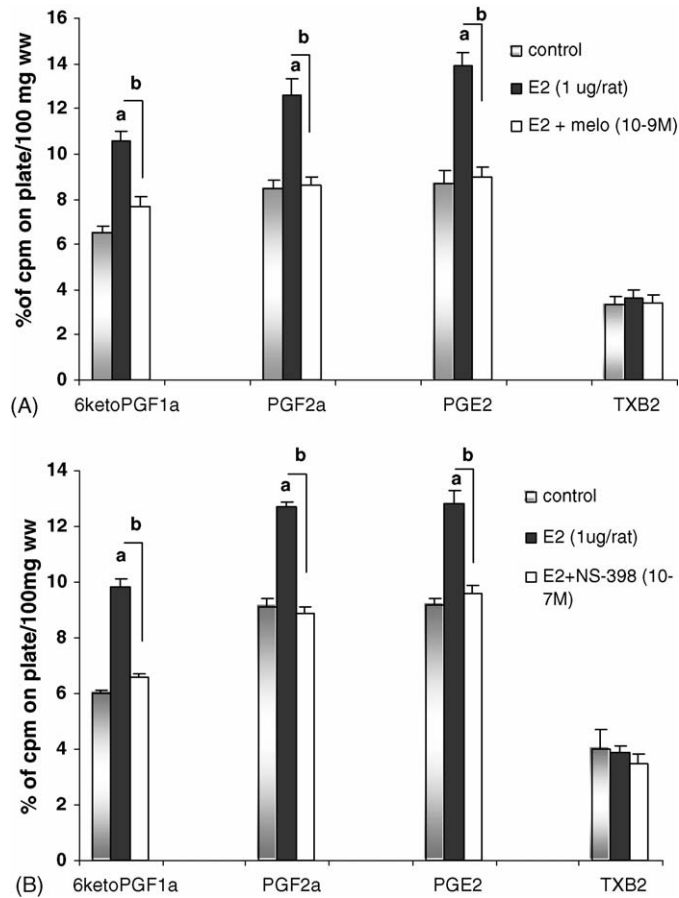
Fig. 6. Effect of *in vitro* treatment with COX-2 selective inhibitors on prostaglandin synthesis after *in vivo* administration of 17-β-estradiol. Oviducts from rats injected with E_2 (1 μg/rat) were incubated in presence of (A) Meloxican (Melo: 10^{-9} M) or (B) NS-398 (10^{-7} M). Control animals were injected with vehicle (C: control). Each bar represents the mean ± S.E.M. of triplicate determinations from four representative animals. Values are expressed as mean ± S.E.M. (a) $p < 0.01$ vs. C; (b) $p < 0.01$ E_2 vs. E_2 + Melo in panel (A). (a) $p < 0.01$ vs. C $p < 0.01$, (b) E_2 vs. E_2 + NS-398 in panel (B).

abolished the increase in PGs synthesis due to 17-β-estradiol (Fig. 6A). This result suggested the participation of the COX-2 isoform in the 17-β-estradiol-induced PGs synthesis. However, the pre-incubation with meloxicam was not able to modify basal PGs production, suggesting that those eicosanoids may become from COX-1 activity.

To confirm these data we tested another COX-2 selective inhibitor, NS-398 (10^{-7} M) [32]. As shown in Fig. 6B, the stimulatory effect of 17-β-estradiol was greatly reduced in the presence of NS-398, while as we have previously observed with meloxican, the basal PGs synthesis was not modified.

4. Discussion

In the mammalian oviduct, important events leading to the establishment of pregnancy take place. These events include final maturation and transport of the female and male gametes, fertilization, early cleavage-stage embryonic development, and transport of the embryo to the uterus. These processes must be well regulated to maximize the chance of fertilization and to allow a successful embryo transport to the uterus.

Our findings reveal, for the first time, that rat oviduct expressed both COX-1 and COX-2 and that only COX-2 expression was modulated in the estrous cycle, showing the highest expression in proestrous and estrous stages. For this reason, we studied the effect of steroid hormones on COX-2 expression. We found that although the administration of estradiol increased COX-2 protein, progesterone had no effect. These results suggest that estradiol

could be up-regulating COX-2 levels in the oviduct during the pre-ovulatory period, in coincidence with the LH surge.

Results from immunohistochemistry indicated that COX-2 was highly expressed in luminal epithelial cells and weakly in vascular endothelial cells from oviducts of estrogenized rats. It has been suggested that the co-localization of COX-2 or COX-1 with prostacyclin synthase (PGIS) near the RER or the nuclear envelope of oviductal epithelial cells may enhance functional coupling of COX with PGIS, thus allowing PGI_2 synthesis [22]. Since we observed COX-2 positive stain mainly in the perinuclear region, we hypothesized that it may be near the endoplasmic reticulum and the Golgi apparatus of epithelial cells, possibly allowing a functional coupling between these two proteins in the rat oviduct.

Cyclooxygenase-2 expression increased by E_2 was partially inhibited by tamoxifen, suggesting that the action of E_2 could be receptor-mediated.

Our results from western blotting studies indicated that, in the rat oviduct, COX-2 is constitutively expressed. However, this could not be observed by immunohistochemistry, probably due to the low sensibility of the assay. Recent reports indicate that COX-2 is constitutively expressed in human [21] and murine oviducts [22].

We also found that estradiol treatment increased PGs (6 keto $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGE_2) production in the oviductal tissue and that this effect was inhibited by two selective enzyme inhibitors, meloxican and NS-398. These results, together with the finding that COX-1 was not regulated during the estrous cycle in the oviduct, confirm that the enhancement of PGs synthesis by E_2 involves COX-2 isoform.

On the other hand, treatment with tamoxifen produced a significant inhibition of PGs synthesis with respect to oviducts from E_2 treated rats, suggesting that the steroid effect on COX-2 activity was receptor specific. In addition, tamoxifen plus E_2 treatment inhibited PGs synthesis even below control levels.

We suggest that, in the rat oviduct, estradiol could increase COX-2 expression thus stimulating PGs synthesis. Another possibility is that the treatment with estradiol could affect both the expression and the activity of COX-2 enzyme at the same time. Also, we could not discard that E_2 was regulating the expression of COX-2 gene.

The results presented in this work are coincident with the report of Wijayagunawardane et al. [8] who suggested that the pre-ovulatory LH surge, together with the increasing E_2 secretion, stimulates the oviductal VEGF system, thus inducing PGs production in bovine oviductal epithelial cells.

Several authors demonstrated that the expression of COX-2 and the synthesis of PGs are regulated by estradiol in different reproductive tissues. Estradiol upregulates COX-2 mRNA expression in myometrial and in human uterine microvascular endothelial cells (HUMEC) [33], and stimulates prostaglandin synthesis in the uterus of non-pregnant rats [34]. Estradiol has also been shown to inhibit PGE_2 production after the suppression of COX-2 induction during pregnancy in the rabbit [35]. It has been also demonstrated that COX-2 regulation by E_2 could be tissue–specific [36].

Oviductal functions depend on the regulation of smooth muscle contractility, ciliary beats of the epithelial cells and the synthesis of oviduct-derived factors. Prostaglandins are known to regulate different functions in the oviduct. Prostacyclin acts in a paracrine fashion relaxing vascular smooth muscle and may play an important role in the initiation of embryos transit through the isthmus of the fallopian tube [21]. Prostacyclin is important in other oviductal functions, such as gametes performance and embryo development [22].

Local production of prostaglandin E_2 and $F_{2\alpha}$ as well as the regulation of oviductal functions have been extensively studied. Prostaglandins are involved in oviductal contraction–relaxation, i.e PGs from the E-series relax the smooth muscle while those from the F-series contract it [37]. The contractions of the circular or the longitudinal tubal muscle are inhibited and stimulated by PGE_2 possibly through four different PGE_2 receptors [21]. $PGF_{2\alpha}$ is mainly involved in oviductal contraction and it is an important factor in the maintenance of the basal tubal tone [38].

Results presented in this work suggest that an increase in E_2 secretion during the pre-ovulatory period would upregulate oviductal PGs production leading to an active oviduct contraction–relaxation effect, therefore allowing gametes transport to the fertilization site. Thus, these prostanoids together with other factors, such as endothelin, cytokines or growth factors would be regulating embryo transport to the uterus.

In summary, the rat oviduct expressed COX-2 enzyme which was positively regulated by estradiol. The synthesis of PGI_2, PGE_2 and PGF_{2a} was increased by this steroid suggesting that prostanoid system is finely regulated in order to ensure oviductal functions. Further studies are required to establish which other factors are involved in the regulation of COX-2 expression and/or prostanoid synthesis during fertilization and embryo transport in the rat oviduct.

Acknowledgements

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