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Multiple Female Reproductive Failures in Cyclooxygenase 2–Deficient Mice

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Summary

Cyclooxygenase (COX) is the rate-limiting enzyme in the synthesis of prostaglandins (PGs) and exists in two isoforms, COX-1 and COX-2. In spite of long-standing speculation, definitive roles of PGs in various events of early pregnancy remain elusive. We demonstrate herein that the targeted disruption of *COX-2*, but not *COX-1*, in mice produces multiple failures in female reproductive processes that include ovulation, fertilization, implantation, and decidualization. Using multiple approaches, we conclude that these defects are the direct result of target organ–specific COX-2 deficiency but are not the result of deficiency of pituitary gonadotropins or ovarian steroid hormones, or reduced responsiveness of the target organs to their respective hormones.

Introduction

Cyclooxygenase (COX), which exists in two isoforms, COX-1 and COX-2, is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs). COX mediates the conversion of arachidonic acid into PGH_2, which is then converted to various PGs by specific synthases (Smith and DeWitt, 1996). COX-1 and -2 are encoded by two separate genes and exhibit distinct cell-specific expression, regulation, and subcellular localization, yet share similar structural and kinetic properties. COX-1 is a constitutive enzyme and is associated with the endoplasmic reticulum (ER). PGs synthesized in the ER by COX-1 exit cells and function via G protein–coupled cell surface receptors to mediate "housekeeping" functions. In contrast, COX-2 is an inducible enzyme and is primarily associated with the nuclear envelope, suggesting a direct effect on nuclear events. Possible effects of COX-2 at the nuclear level are reinforced by observations that various fatty acids and PGs can function as ligands for nuclear peroxisome proliferator activated receptors (PPARα, PPARγ, and PPARδ) (Forman et al., 1995, 1997; Kliewer et al., 1995, 1997). This supports the concept that distinct subcellular localization of COX-1 and COX-2 is associated with their compartmentalized functions (Morita et al., 1995; Smith and DeWitt, 1996). COX-2 is induced in a variety of cell types by diverse stimuli including cytokines, growth factors, mitogens, and tumor promoters (Kujubu et al., 1991; Lee et al., 1992; DeWitt and Meade, 1993; Ristimaki et al., 1994; Coffey et al., 1997). Since COX-2 is primarily responsible for increased PG production during inflammation, this isoform is the target for development of selective anti-inflammatory drugs (Copeland et al., 1994; Pinto et al., 1995; Kurumbail et al., 1996). COX-2 overexpression is also associated with tumorigenesis (DuBois et al., 1996; Oshima et al., 1996).

Ovulation is a process that depends upon the coordinated effects of pituitary gonadotropins that mediate follicular development, oocyte maturation, and rupture of antral follicles (Tsafriri and Chun, 1996). The postovulatory follicles transform into corpora lutea, which produce progesterone (P_4) to support pregnancy following a timely interaction between an ovulated egg and a sperm that leads to successful fertilization. Synchronized development of the embryo to the blastocyst stage and differentiation of the uterus to the receptive state are essential to the implantation process (Psychoyos, 1973; Paria et al., 1993). In the mouse, the establishment of a receptive uterus for supporting implantation is regulated by coordinated effects of P_4 and estrogen (Huet-Hudson et al., 1989). Increasing levels of P_4 from newly formed corpora lutea from day 3 of pregnancy directs stromal cell proliferation, which is further potentiated by ovarian estrogen secretion in the morning of day 4. In contrast, the luminal epithelium becomes differentiated on day 4 for interactions with the blastocyst during the attachment reaction, which occurs at 2200–2300 hr on this day. The first conspicuous sign of the implantation process is an increased endometrial vascular permeability at the sites of blastocyst apposition, and this coincides with the initial attachment reaction (Psychoyos, 1973; Paria et al., 1993). The attachment reaction is followed by adherence and penetration by trophoblast cells through the underlying basement membrane and results in proliferation and differentiation of stromal cells into decidual cells (decidualization) (Dey, 1996). Ovariectomy prior to preimplantation ovarian-estrogen secretion on day 4 results in blastocyst dormancy and failure in the attachment reaction, a condition termed delayed implantation. This condition can be maintained by continued P_4 treatment and terminated by an injection of estrogen with blastocyst activation and initiation of the attachment reaction (Yoshinaga and Adams, 1966; Paria et al., 1993).

Vasoactive, mitogenic and differentiating properties of PGs (Smith and DeWitt, 1996) have been implicated in various female reproductive functions. The processes of ovulation and implantation are considered analogous to "proinflammatory" responses, and thus participation of PGs in these processes are speculated (McMaster et al., 1993; Espey, 1994). For example, PGs are considered to participate in follicular rupture during ovulation (reviewed in Tsafriri and Chun, 1996). This is consistent with gonadotropin-mediated induction of COX-2 in ovarian follicles preceding ovulation (Sirois et al., 1992; Sirois,

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1994). PGs are also implicated as important mediators of increased endometrial vascular permeability during implantation and decidualization (reviewed in Chakraborty et al., 1996). Unique expression of *COX-1* and *COX-2* genes in the peri-implantation mouse uterus further supports the conclusion that PGs play important roles in these processes (Chakraborty et al., 1996). In this study, we observed that COX-1 is expressed in uterine epithelial cells in the morning of day 4 of pregnancy, but the expression becomes undetectable by the time of the attachment reaction. In contrast, COX-2 is expressed in the luminal epithelium and underlying stromal cells solely at the sites of blastocyst attachment reaction. Employing the delayed implantation model, this study also established that the expression of COX-2 in the receptive uterus requires the presence of active blastocysts. The results suggested that COX-2 expression during the attachment reaction is critical for implantation. However, the definitive roles of COX-1 and/or COX-2 in these reproductive functions require animal models in which *COX-1* or *COX-2* genes have been mutated.

COX-1- and COX-2-deficient mice were recently generated by gene targeting (Dinchuk et al., 1995; Langenbach et al., 1995; Morham et al., 1995). COX-1 homozygous mutant females are apparently fertile with limited parturition defects. In contrast, absence of COX-2 resulted in female infertility. Based on limited results, defective ovulation was considered as the cause of this infertility, although follicular development was apparently normal. However, the importance of uterine COX-2 deficiency was not explored. To delineate roles of COX-2 in early pregnancy, we used COX-1- and COX-2-deficient mice to examine the consequences of COX-2 deficiency in ovulation, fertilization, implantation, and decidualization. Our results establish that COX-2 is essential for these processes.

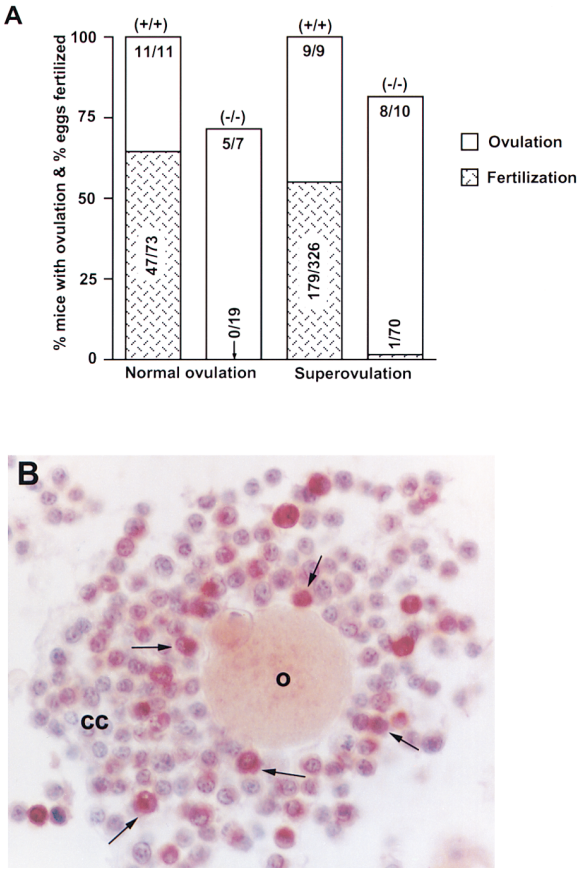

Figure 1. Ovulation and Fertilization in *COX-2*(−/−) Mice

(A) Rates of ovulation and fertilization in *COX-2*(+/+) or *COX-2*(−/−) mice were examined on days 1 and 2 of pregnancy with or without superovulation. The numbers within the empty bars indicate the number of mice with ovulation/total number of mice, and the numbers within the hatched bars indicate the number of fertilized eggs/total number of ovulated eggs.

(B) A representative photomicrograph of COX-2 immunostaining in wild-type cumulus cell–enclosed ovulated egg (o). Arrow indicates perinuclear localization of COX-2 in cumulus cells (cc). Magnification, 240×.

Results

Ovulation and Fertilization Are Defective in COX-2-Deficient Mice

COX-2(−/−) female mice are infertile and this was attributed to ovulation failure in spite of apparently normal follicular development (Dinchuk et al., 1995). We reexamined in more detail the ovulation and subsequent events in these mice. As shown in Figure 1A, 64% (47/73, n = 11) of eggs recovered from wild-type females were fertilized. In contrast, only 19 eggs were recovered from 5 of 7 *COX-2*(−/−) females and none of them was fertilized. These eggs were developmentally abnormal, since extrusion of the first polar body was rarely observed. The results establish that *COX-2*(−/−) females have defective ovulation and complete failure of fertilization. We surmised that these defects could be due to deficiency of pituitary gonadotropins and/or ovarian responsiveness to gonadotropins. Thus, *COX-2*(−/−) females were subjected to superovulation with gonadotropins. As shown in Figure 1A, an average of 36 eggs/mouse were recovered from all of the wild-type mice (n = 9) examined, while an average of only 9 eggs/mouse were recovered from 8 of 10 *COX-2*(−/−) mice. Although the number of ovulation was slightly improved in gonadotropin-stimulated *COX-2*(−/−) mice, the presence of numerous developing follicles suggested that the ovaries responded to gonadotropins (data not shown). Again, fertilization was severely compromised in *COX-2*(−/−) mice [1.4% in *COX-2*(−/−) versus 55% in wild-type]. These results establish that the reduced rate of ovulation was not the result of deficiency of pituitary gonadotropins. Further, the presence of numerous sperm in the oviduct suggested that the fertilization failure was not due to the absence of sperm at the site of fertilization (data not shown). Collectively, these results establish that COX-2 deficiency is the major cause of defective ovulation and fertilization. The induction of COX-2 in ovarian follicles by gonadotropins (Sirois et al., 1992; Sirois, 1994) is also consistent with this conclusion. The cause of fertilization failure in *COX-2*(−/−) females could be due to impaired oocyte maturation. This is supported by our observation of perinuclear accumulation of COX-2

Table 1. Implantation of Wild-Type Blastocysts Transferred to Pseudopregnant *COX-2*(+/+) or *COX-2*(−/−) Mice

Genotype	No. of Blastocysts Transferred	No. of Recipients	No. of Mice with IS	No. of Mice without IS	No. of IS (%)	No. of Blastocysts Recovered
(+/+)	84	5	4	1	42 (50)	1
(−/−)	156	9	2	7	2 (1.3)	45 (11[a])

Day 4 wild-type blastocysts were transferred into uteri of *COX-2*(+/+) or *COX-2*(−/−) mice on day 4 of pseudopregnancy. Recipients were killed on day 5 to examine implantation sites (IS) by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted blastocysts.

[a] Indicates number of degenerating blastocysts.

in cumulus cells surrounding oocytes in antral follicles and ovulated eggs of wild-type mice (Figure 1B).

Wild-Type Blastocysts Fail to Implant in Uteri of *COX-2*(−/−) Mice

Since COX-2 is expressed uniquely in the mouse uterus surrounding the implanting blastocyst (Chakraborty et al., 1996), we examined whether uterine COX-2 deficiency impedes the implantation process. However, this could not be examined in normally mated *COX-2*(−/−) females because of defective ovulation and severely impaired fertilization. To circumvent these problems, we employed blastocyst transfer experiments. In these experiments, wild-type day 4 blastocysts were transferred into uteri of day 4 pseudopregnant wild-type or *COX-2*(−/−) mice, and implantation sites were recorded on day 5 by the blue dye method (Paria et al., 1993). While 50% (42/84) of the transferred blastocysts implanted in 80% (4/5) of the wild-type recipients, only 1.3% (2/156) of transferred blastocysts implanted in 22% (2/9) of *COX-2*(−/−) recipients (Table 1). The recovery of 45 blastocysts from uteri of 7 *COX-2*(−/−) females not exhibiting implantation indicated successful blastocyst transfer in these mice, but the failure of the attachment reaction. These results suggest that the implantation process in the COX-2-deficient uterus is defective.

In the mouse, secretion of P_4 from newly formed corpora lutea accompanied by preimplantation ovarian estrogen secretion on day 4 morning is critical for the establishment of uterine receptivity for implantation. Uterine receptivity occurs only for a limited time during pregnancy or pseudopregnancy on day 4, and is preceded or followed by prereceptive or nonreceptive state on days 3 or 5, respectively (Dey, 1996). Blastocysts transferred into a nonreceptive uterus fail to implant and degenerate (Paria et al., 1993). We reasoned that the failure of wild-type blastocysts to implant after transfer into pseudopregnant *COX-2*(−/−) uteri could be due to insufficient P_4 secretion from too few corpora lutea resulting from a reduced rate of ovulation. To address this issue, we used a delayed implantation model where the wild-type or *COX-2*(−/−) recipients were ovariectomized on day 4 of pseudopregnancy and given daily injections of P_4 on days 5–7. On day 7, wild-type blastocysts were transferred into uteri of these mice followed by an injection of estradiol-17β (E_2) to induce implantation (Paria et al., 1993). As shown in Table 2, 16/47 (34%) transferred blastocysts implanted in uteri of all wild-type recipients (n = 4) examined. In contrast, none of the transferred blastocysts (n = 82) implanted in *COX-2*(−/−) recipients (n = 5); 24 unimplanted blastocysts were recovered from uteri of these mice. The results demonstrate that inadequacy of ovarian steroids was not the cause of implantation failure, attesting again to the critical role of COX-2 in implantation.

Selective COX-2 Inhibitor Interferes with Implantation in Wild-Type or COX-1-Deficient Mice in a Dose-Dependent Manner

To further confirm the role of COX-2 in implantation, we sought to simulate a *COX-2*(−/−) phenotype with respect to implantation by inhibiting COX-2 activity in wild-type and *COX-1*(−/−) mice. DuP697 was used as a selective inhibitor of COX-2 (Copeland et al., 1994), while acetylsalicylic acid (aspirin) was used as a preferential inhibitor of COX-1 (Mitchell et al., 1994; Pinto et al., 1995). Administration of DuP697 on days 3 and 4 of pregnancy resulted in a dose-dependent inhibition of implantation in wild-type, *COX-1*(+/−), or *COX-1*(−/−) mice. DuP697 at a dose of 120 nmol/injection did not adversely affect implantation in either wild-type or

Table 2. Implantation of Wild-Type Blastocysts in Delayed Pseudopregnant *COX-2*(+/+) or *COX-2*(−/−) Mice

Genotype	No. of Blastocysts Transferred	No. of Recipients	No. of Mice with IS	No. of Mice without IS	No. of IS (%)	No. of Blastocysts Recovered
(+/+)	47	4	4	0	16 (34)	–
(−/−)	82	5	0	5	0	24 (9[a])

Conditions of delayed implantation in pseudopregnant mice were induced by ovariectomy on the morning of day 4 and maintained by daily P_4 (2 mg/mouse) injections (day 5–7). Day 4 wild-type blastocysts were transferred into uteri of these delayed recipients on day 7 and an injection of E_2 (25 ng/mouse) was given to terminate the conditions of delayed implantation and to initiate implantation. Recipients were killed on day 8 to examine implantation sites (IS) by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted blastocysts.

[a] Indicates number of degenerating blastocysts.

Table 3. Effects of DuP697 or Acetylsalicylic Acid (Aspirin) on implantation in *COX-1*(+/+), *COX-1*(+/−), or *COX-1*(−/−) Mice

Genotype	Treatment (nmol/injection)	No. of Mice	No. of Mice with IS	No. of Mice without IS	No. of IS	No. of Blastocysts Recovered
(+/+)(+/−)	vehicle	6	6	0	57	
(−/−)	vehicle	4	4	0	40	
(+/+)(+/−)	DuP697 (120)	7	7	0	52	
(−/−)	DuP697 (120)	12	11	1	61(12[a])	8
(+/+)(+/−)	DuP697 (600)	8	4	4	16[a]	28
(−/−)	DuP697 (480)	6	1	5	2[a]	38
(+/−)	Aspirin (700, 1400)	12	12	0	118	
(−/−)	Aspirin (700, 1400)	6	6	0	51	

Mice were injected with DuP697 (COX-2 inhibitor) or acetylsalicylic acid (Aspirin, COX-1 inhibitor) on day 3 morning, and morning and evening of day 4 of pregnancy. They were killed on day 5 to record implantation sites (IS) by the blue dye method. Uteri without IS were flushed with saline to recover any unimplanted blastocysts. Mice without IS or blastocysts were not included in computing the results. The results in *COX-1*(+/+) and *COX-1*(+/−) mice were grouped together. Since treatment with two different doses of Aspirin provided similar results, they were grouped together.

[a] IS with weak blue dye reaction.

COX-1(−/−) females. However, implantation was severely compromised in wild-type and *COX-1*(+/−) mice when the dosage was increased to 600 nmol/injection, or in *COX-1*(−/−) mice at 480 nmol/injection (Table 3). Since similar results were obtained with wild-type and *COX-1*(+/−) mice, they were grouped together and used for comparison. Only a total of 16 weak (faint blue reaction) implantation sites from 4 of 8 wild-type/*COX-1*(+/−) mice were observed. Blastocysts were easily recovered by flushing the uteri of these mice with saline, suggesting weak attachment reaction. The remaining 4 mice did not show any implantation sites, but 28 unimplanted blastocysts were recovered. In *COX-1*(−/−) mice, only 1 of 6 mice showed 2 weak implantation sites and a total of 38 unimplanted blastocysts were recovered from the remaining 5 mice without implantation sites. DuP697 was relatively more effective in impeding implantation in the absence of COX-1. In contrast, treatment with acetylsalicylic acid even at 700 or 1400 nmol/injection for days 3 and 4 of pregnancy did not impede implantation in either *COX-1*(+/−) or *COX-1*(−/−) mice (Table 3). These results reinforce the conclusion that COX-2 plays an essential role in implantation.

Decidualization Is Defective in *COX-2*(−/−) Mice

The attachment reaction is followed by extensive stromal cell proliferation and differentiation into decidual cells. The decidualization is preceded by increased endometrial vascular permeability and is initiated at the antimesometrial sites where blastocysts implant. Initially, stromal cells only surrounding the implanting blastocyst proliferate and form the primary decidual zone (PDZ) on day 5. This is followed by proliferation of the stromal cells outside the PDZ by day 6, eventually forming the secondary decidual zone (Dey, 1996). In pregnant mice, the stimulus for decidualization is the implanting blastocyst. This process, however, can be induced artificially in pseudopregnant or steroid hormonally prepared uterus by intraluminal infusion of oil or phosphate-buffered saline (PBS) containing gelatin (Rankin et al., 1977; Kennedy, 1983). This model has been used widely to study the mechanism(s) of decidualization. Although the blastocyst attachment fails to occur in the absence of COX-2, whether this isozyme is also critical for decidualization is not known. To address this question, we examined this process in *COX-2*(−/−) mice by intraluminal oil infusion on day 4 of pseudopregnancy as an inducer of artificial decidualization. The results demonstrate that while 73% (8/11) of the wild-type mice showed an average of 16-fold increase in uterine weight with massive decidualization, none of the *COX-2*(−/−) mice (n = 7) showed this response after intraluminal infusion of oil (Figure 2A). To circumvent any steroid deficiency resulting from defective ovulation, we further examined the decidualization process in ovariectomized mice treated with a well-established steroid hormonal regimen (see Experimental Procedures) that optimizes the uterus for decidualization (Wordinger et al., 1986). While 81% (13/16) of the wild-type mice showed an 8-fold increase in uterine weights, none of the *COX-2*(−/−) mice (n = 9) exhibited this response, again demonstrating failure of decidualization in the absence of COX-2 (Figure 2B). Collectively, the results suggest that both the initial attachment reaction and the subsequent decidualization process are impaired in the absence of COX-2.

COX-2 is an immediate-early gene induced by various stimuli (Smith and DeWitt, 1996). It was reported that uterine levels of PGs and intracellular cAMP rapidly increase after application of a deciduogenic stimulus (Rankin et al., 1977; Kennedy, 1983). Failure of decidualization in *COX-2*(−/−) mice suggested that induction of uterine COX-2 could be important at initial stages of decidualization. To address this issue, we examined the expression of *COX-1* and *COX-2* in the wild-type uterus 2 and 8 hr after intraluminal infusion of oil (deciduogenic stimulus) at 1000 h on day 4 of pseudopregnancy. No *COX-2* mRNA accumulation was noted in the luminal epithelium of the non-infused horn. In contrast, high levels of this mRNA accumulated in the luminal epithelium of the oil-infused horn within 2 h followed by precipitous decline by 8 h (Figures 3A and 3B). Changes in *COX-1* mRNA were minimal after intraluminal oil infusion. It should be recalled that COX-2 is undetectable in the uterine epithelium in the morning of day 4 of pregnancy or pseudopregnancy (Chakraborty et al., 1996). Thus, the rapid induction of luminal epithelial

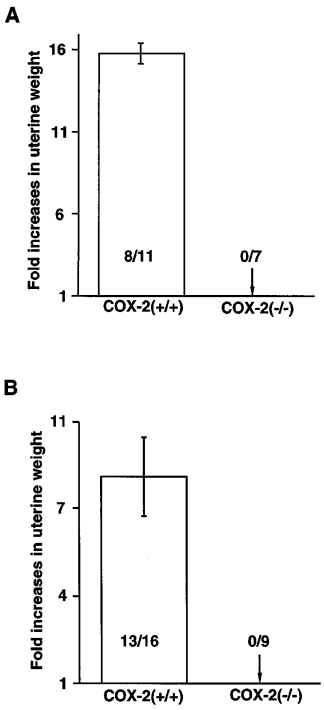

Figure 2. Decidualization in *COX-2*(−/−) Mice

(A) *COX-2*(+/+) or *COX-2*(−/−) mice received intraluminal oil infusion on day 4 of pseudopregnancy. On day 8, mice were killed and uterine weights were recorded. Fold increases denote comparison of weights between infused and noninfused uterine horns.

(B) Ovariectomized *COX-2*(+/+) or *COX-2*(−/−) mice were treated with P_4 and E_2 (see Experimental Procedures), which sensitize the uterus for optimal decidualization. Intraluminal infusion of oil was made on the 8th day of the hormone treatment and mice were killed 4 days later to record the fold increases in uterine weights. Number of responding mice/total number of mice is shown. Results are mean ± SEM.

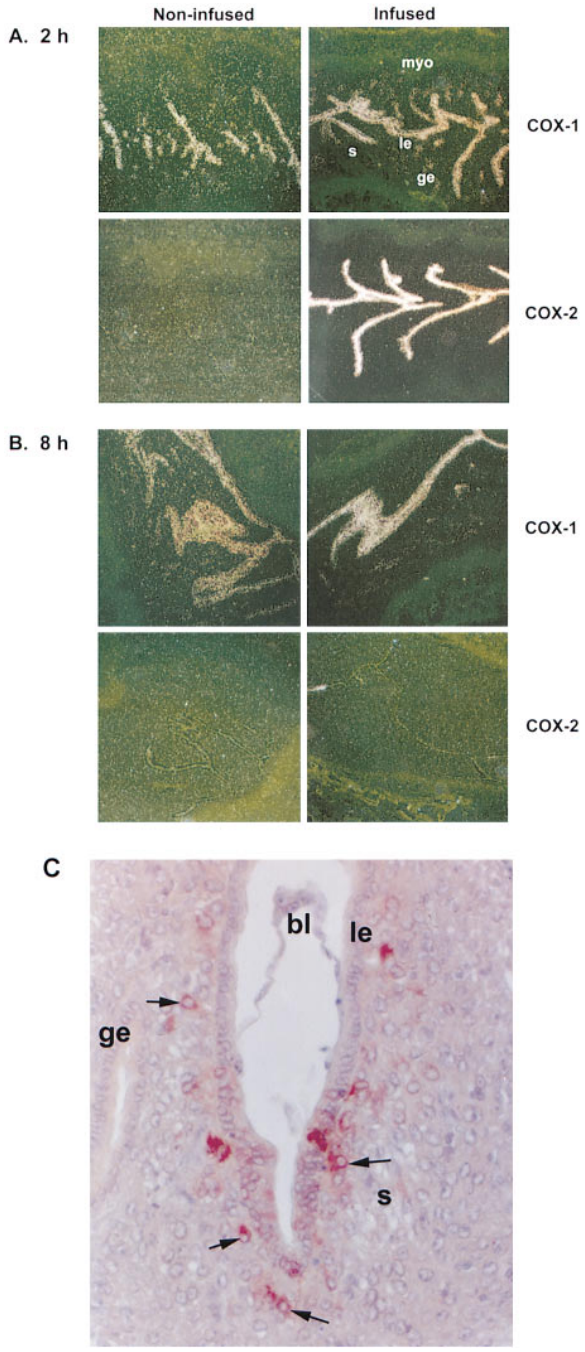

Figure 3. In Situ Hybridization of *COX-1* and *COX-2* mRNAs in Day 4 Pseudopregnant Wild-Type Uterus with or without Intraluminal Oil Infusion

(A) Two hours after oil infusion; (B) 8 hr after oil infusion. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. Magnification, 28×.

(C) Immunostaining of COX-2 in the implantation site on day 5 of pregnancy in the wild-type mice. Arrows indicate perinuclear localization of COX-2 at the site of blastocyst attachment. bl, blastocyst; le, luminal epithelium; ge, glandular epithelium; s, stroma. Magnification, 140×.

COX-2 mRNA in the oil-infused horn is remarkable and points toward its role in the initiation of decidualization, supporting the observation of decidualization failure in *COX-2*(−/−) mice. The rapid induction of uterine COX-2 after the application of the deciduogenic stimulus in wild-type pseudopregnant females is analogous to induction of COX-2 in the uterus by the implanting blastocysts (Chakraborty et al., 1996). It should be noted that the location of the COX-2-positive cells around the implantation chamber is the area that exhibits the first decidual cell reaction (Figure 3C), suggesting its role in this process.

Expression of Implantation-Specific Genes and Steroid Hormone-Responsiveness in the Uterus of *COX-2*(−/−) Mice Are Normal

The results described above demonstrated that impaired implantation and decidualization in *COX-2*(−/−) mice were at least independent of E_2 and P_4. However, we speculated that these defects could be due to aberrant expression of specific genes considered important for implantation. Thus, we examined the expression of leukemia inhibitory factor (*LIF*), *Hoxa-10*, and amphiregulin (*Ar*) in day 4 pregnant uteri of the wild-type or

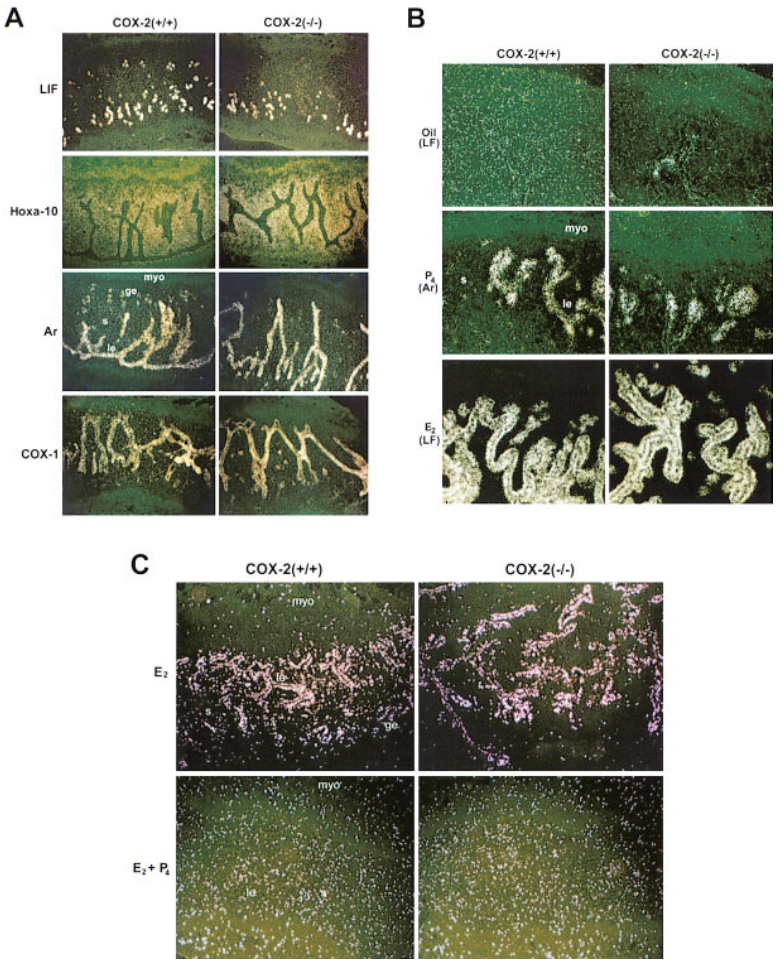

Figure 4. Expression of Implantation-Specific Genes and Uterine Responsiveness to E_2 and/or P_4 in *COX-2*(−/−) Mice

(A) In situ hybridization of *LIF*, *Hoxa-10*, *Ar*, and *COX-1* mRNAs in day 4 pregnant uteri of *COX-2*(+/+) or *COX-2*(−/−) mice. Magnification, 24×.

(B) In situ hybridization of *LF*, an estrogen-responsive gene and *Ar*, a P_4-responsive gene, in the ovariectomized *COX-2*(+/+) or *COX-2*(−/−) mice treated with oil (vehicle), E_2 or P_4. Oil-treated (control) uterine sections did not show any specific autoradiographic signals for either *LF* or *Ar* mRNA; only mRNA for *LF* is shown. E_2-treated mice were killed at 24 hr, while those treated with P_4 were killed at 6 hr. Magnification, 24×.

(C) Nuclear incorporation of [^{3}H]thymidine in the ovariectomized uteri of *COX-2*(+/+) or *COX-2*(−/−) mice after administration of E_2 or E_2 + P_4. Note nuclear thymidine uptake in epithelial cells after E_2 treatment and in stromal cells after E_2 + P_4 treatment. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium. Magnification, 24×.

COX-2(−/−) mice. LIF is expressed in uterine glandular epithelia on day 4 and abrogation of this gene function by homologous recombination causes implantation failure (Stewart et al., 1992). Likewise, absence of *Hoxa-10*, an *Abdominal B* (*AbdB*) class homeobox gene, in female mice causes peri-implantation failure (Satokata et al., 1995; Benson et al., 1996). Ar, a member of the EGF superfamily, is expressed in an implantation-specific manner and is regulated exclusively by P_4 in the mouse uterus (Das et al., 1995). We also examined the expression of *COX-1* gene in day 4 pregnant uterus to determine whether COX-2 deficiency altered the expression of this gene. As shown in Figure 4A, results of in situ hybridization experiments did not reveal any aberrant spatiotemporal expression of these genes in *COX-2*(−/−) uteri, suggesting that implantation and decidualization defects were not the result of abnormal expression of these genes.

We still argued that altered responsiveness of the uterus to E_2 and/or P_4 could be associated with uterine defects in *COX-2*(−/−) mice. This issue was addressed using ovariectomized wild-type and *COX-2*(−/−) mice treated with E_2 or P_4. Lactoferrin (*LF*), an estrogen-responsive gene and *Ar*, a P_4-responsive gene, served as established markers for uterine responsiveness to these steroids in the mouse uterus (McMaster et al., 1992; Das et al., 1995). No aberrant expression of these genes was noted, implying *COX-2*(−/−) females respond normally to ovarian steroids with respect to these genes (Figure 4B). During the peri-implantation period, uterine cell–specific proliferation and differentiation are regulated by the coordinated effects of estrogen and P_4. These responses can be reproduced in ovariectomized mice by exogenous administration of these steroids. For example, E_2 stimulates proliferation of luminal and glandular epithelial cells, while in stroma, this process requires both P_4 and E_2 (Huet-Hudson et al., 1989). Thus, we examined whether normal uterine cell–specific proliferation and differentiation occur in *COX-2*(−/−) mice in response to E_2 and/or P_4. Cell-specific nuclear [^{3}H]thymidine incorporation in the uterus was probed by autoradiography as an index of DNA synthesis and cell proliferation. As in wild-type mice (Huet-Hudson et al., 1989), nuclear thymidine uptake in *COX-2*(−/−) uteri was stimulated in epithelial cells by E_2, while a combined treatment with P_4 and E_2 stimulated the nuclear uptake in stromal cells (Figure 4C). Very little or no nuclear thymidine uptake was noted in uteri of ovariectomized *COX-2*(−/−) mice in the absence of these steroids (data not shown). Collectively, these results establish that implantation and decidualization failures in *COX-2*(−/−) mice are not the results of aberrant expression of implantation-specific genes or defective responsiveness of the uterus to steroid hormones. Thus, we conclude

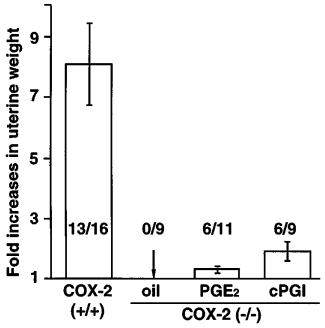

Figure 5. Effects of PGE_2 or cPGI on Decidualization in *COX-2*(−/−) Mice

Ovariectomized *COX-2*(−/−) mice were steroid hormonally prepared as indicated in the legend to Figure 2. Fold increases in uterine weights (infused versus noninfused) after infusion of PGE_2 or cPGI (1 μg/50 μl oil) are shown as compared to those of vehicle (50 μl oil) infused (controls) uterine horns of *COX-2*(+/+) and *COX-2*(−/−). Number of responding mice/total number of mice is shown. Results are mean ± SEM.

that uterine COX-2 plays a critical role in these processes.

Exogenous Administration of PGI_2 Partially Restores Decidualization in COX-2-Deficient Mice

We hypothesized that if PGs derived by COX-2 are important for decidualization, we may be able to correct this defect by administration of PGs. Since PGE_2 has been considered as an enhancer of decidualization in the rat (Kennedy, 1985), in our initial experiments PGE_2 (1 μg/50 μl oil) was infused intraluminally as a deciduogenic stimulus in ovariectomized steroid hormonally prepared *COX-2*(−/−) mice (see Experimental Procedures). Very little or no decidual response was noted in these mice (Figure 5). These results suggest that PGE_2 is either ineffective or has only limited effects in inducing decidualization in the mouse. Using GC/MS, we recently observed that the most abundant PG in the implantation sites of day 5 pregnant wild-type mice is PGI_2. Its level is more than 4-fold higher than those of PGE_2, $PGF_{2\alpha}$, PGD_2, and thromboxane B_2 (unpublished data). Thus, we speculated that PGI_2 may be involved in implantation and decidualization. Since PGI_2 is extremely unstable, carbaprostacyclin (cPGI), a more stable analog of PGI_2, was delivered intraluminally (1 μg/ 50 μl oil) as a deciduogenic stimulus in steroid hormonally prepared ovariectomized *COX-2*(−/−) mice. Approximately a 2-fold induction of decidualization was noted in 67% of the mice infused with cPGI (Figure 5). Day 4 pseudopregnant *COX-2*(−/−) mice similarly infused with cPGI also produced a 2.6-fold induction of decidualization (data not shown). Although this agonist provoked better decidual response than PGE_2, the response was still modest. The inefficiency of exogenously administered PGs to induce optimal decidualization could be due to the their unstable nature, rapid degradation, and/or ineffective delivery to the site of action. Since COX-2 is primarily localized in the nuclear envelope, intranuclear generation of PGs is perhaps the trigger for initiation of decidualization. Therefore, the difficulties in targeting exogenously administered PGs in vivo to the nuclear compartment could be one of the primary reasons for their limited effectiveness in restoring decidual response in *COX-2*(−/−) mice. Alternatively, other PGs or a novel PG(s) generated by COX-2 could be involved in this process. It is also possible that an aberrant expression of cell surface PGE_2 receptors may be the cause of failure of this ligand in inducing decidualization in *COX-2*(−/−) mice.

PGE_2 Receptor Subtypes Are Correctly Expressed in COX-2-Deficient Mice

Signaling by PGE_2 is mediated by a set of G protein–coupled cell surface receptors (EP_1, EP_2, EP_3, and EP_4) that are linked to distinct intracellular signaling pathways (Negishi et al., 1995). We have shown previously that EP_2, EP_3, and EP_4 receptor subtypes are uniquely expressed in the peri-implantation mouse uterus (Yang et al., 1997; Lim and Dey, 1997), suggesting their role in peri-implantation events. Thus, we examined the status of *EP_2*, *EP_3*, and *EP_4* mRNAs in day 4 pregnant uteri of wild-type and *COX-2*(−/−) mice. We noted that these receptor subtypes are appropriately expressed in *COX-2* (−/−) uteri (Figure 6), suggesting that the decidualization failure was not associated with aberrant expression of these receptors.

Cholera Toxin Partially Induces Decidualization in COX-2-Deficient Mice

Cholera toxin (CTX) has been used as an inducer of decidualization in the mouse and rat uterus (Rankin et al., 1977; Kennedy, 1983). It was suggested that increases in intracellular cAMP were responsible for this response. It was also implicated that PGE_2 enhances the decidual response via cAMP (Kennedy, 1983). Thus, we examined whether this toxin infused intraluminally would induce decidualization in *COX-2*(−/−) mice. As shown in Figure 7A, intraluminal infusion of CTX, indeed, induced a 4-fold increase in the decidual response in *COX-2*(−/−) mice compared with no induction in vehicle-treated (PBS-gelatin) *COX-2*(−/−) mice. However, the extent of decidualization in these mice by CTX was not comparable to that observed in wild-type mice, which showed a 13-fold increase in uterine weights by CTX. We reasoned that if CTX is working via raising intracellular cAMP, forskolin having direct stimulatory effects on adenylyl cyclase should produce the same effects as did CTX. Surprisingly, forskolin was without any effect in this response (data not shown). This raises doubts regarding the presumed role of cAMP in decidualization. This is consistent with previous findings of failure of dibutyryl-cAMP in inducing decidualization in the mouse and rat (Rankin et al., 1977; Kennedy, 1983). Differential effects of CTX and forskolin in other cell systems are also documented (Pike and Eakes, 1987; DuBourdieu and Morgan, 1990). Since CTX has been shown to stimulate PG synthesis in other cell types via mobilization of cellular arachidonic acid pool (reviewed in Peterson et al., 1996), we also thought that partial stimulation of decidualization by CTX might have occurred in *COX-2*(−/−) mice through increased PG synthesis via COX-1. However, the failure of indomethacin

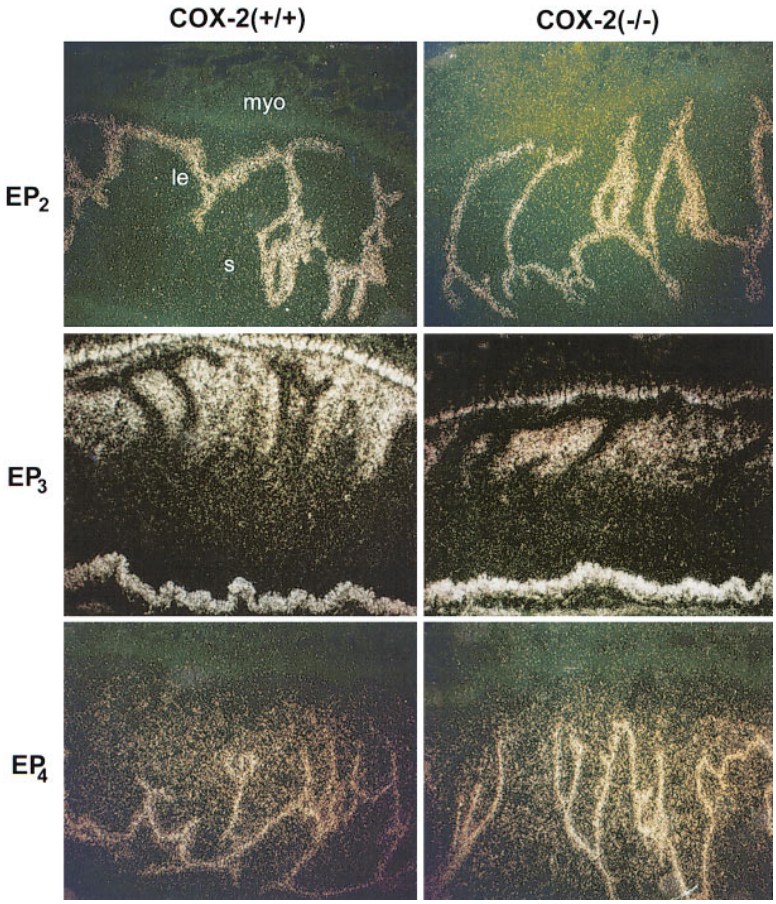

Figure 6. Expression of PGE_2 Receptor Subtypes in *COX-2*(−/−) Mice

In situ hybridization of EP_2, EP_3, and EP_4 mRNAs in day 4 pregnant uteri of *COX-2*(+/+) or *COX-2*(−/−) mice is shown. le, luminal epithelium; s, stroma; myo, myometrium.

(a preferential COX-1 inhibitor) to block CTX-induced decidualization in *COX-2*(−/−) mice (data not shown), as well as unaltered expression patterns of *COX-1* mRNA before and after intraluminal infusion of CTX in *COX-2* (−/−) mice (Figure 7B), is not consistent with this hypothesis. In contrast, our observations of intense decidualization accompanied by remarkable induction of uterine *COX-2* mRNA after either oil (Figure 3A) or CTX (Figure 7C) infusion in wild-type mice suggest that normal decidualization would require the induction of uterine COX-2. CTX-induced modest decidualization observed in *COX-2*(−/−) mice could, however, be mediated by a mechanism not yet defined.

Discussion

The pathophysiological significance of PGs in various female reproductive functions have been studied for decades by examining the effects of exogenously administered PGs and/or pharmacological inhibitors of PG synthesis. Although these studies provided circumstantial evidence, the definitive roles of PGs in female reproduction still remain undefined. Identification of two distinct COX isoforms in PG synthesis at distinct subcellular localizations offers further challenge as to which of the COX pathways is critical to various reproductive functions. The normal and adaptive functioning of cells requires the coordinated expression of "housekeeping" and "inducible" genes. In this respect, PGs generated by COX-1 are considered important for maintenance of many housekeeping functions, while those generated by COX-2 are thought to participate in inducible functions (Smith and DeWitt, 1996). During early pregnancy, many of the inducible events include ovulation, fertilization, implantation, and decidualization. The inducible nature of COX-2 suggests that participation of PGs in these processes is likely to involve this isoform. Indeed, we clearly establish herein that timely and target-specific expression of COX-2 is essential for several reproductive and developmental events that include ovulation, fertilization, implantation, and decidualization. In contrast, these processes are apparently normal in COX-1-deficient mice (Langenbach et al., 1995), suggesting that COX-1 is not essential for these events.

Differential subcellular sites of PG production by COX-1 and COX-2 may account for distinct roles of these two isoforms in various cellular functions. PGs mediate their functions via cell surface or nuclear receptors (Negishi et al., 1995; Forman et al., 1995, 1997; Kliewer et al., 1995, 1997). PGs produced by COX-1 in the ER are likely to be secreted and to interact with cell surface receptors in an autocrine or paracrine manner, while PGs produced by COX-2 in the nuclear envelop are likely to interact with nuclear receptors in an intracrine fashion. The observations that these two isoforms utilize different phospholipase systems and lipid pools for conversion of arachidonic acid to PGs (Reddy and Herschman, 1997) are consistent with compartmentalized functions of these two isoforms.

The participation of COX-2 in ovulation was suggested

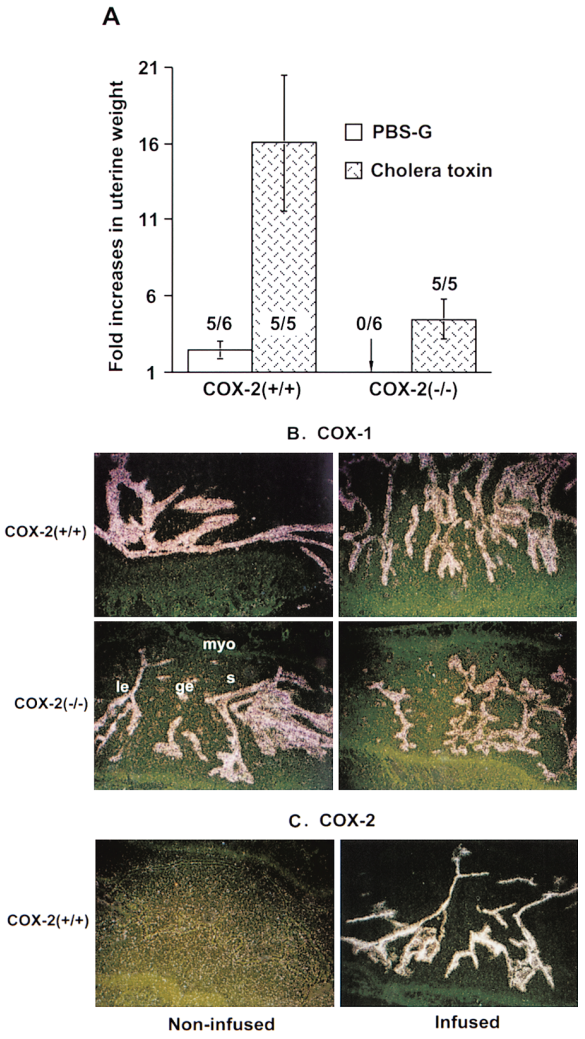

Figure 7. Effects of Cholera Toxin (CTX) on Decidualization in *COX-2* (−/−) Mice

(A) Ovariectomized *COX-2*(+/+) or *COX-2*(−/−) mice were steroid hormonally prepared as indicated in the legend to Figure 2. Fold increase in uterine weights (infused versus noninfused horns) after intraluminal infusion of CTX (50 ng/50 μl) are shown as compared to vehicle (50 μl PBS containing 0.1% gelatin) infusion. Number of responding mice/total number of mice is shown. Results are mean ± SEM.

(B) In situ hybridization of *COX-1* mRNA in day 4 pseudopregnant uteri of *COX-2*(+/+) or *COX-2*(−/−) mice 2 hr after intraluminal infusion of CTX. Magnification, 24×.

(C) In situ hybridization of *COX-2* mRNA in day 4 pseudopregnant *COX-2*(+/+) uteri 2 hr after intraluminal infusion of CTX. Magnification, 24×.

because of the rapid but transient induction of this isoform in granulosa cells after gonadotropin stimulation (Sirois et al., 1992; Sirois, 1994). Severely compromised ovulation with apparently normal follicular development in COX-2-deficient mice even after a superovulatory stimulus suggests that the absence of COX-2 in the ovary, but not gonadotropin deficiency or defective responsiveness of the ovary to these hormones, is the primary cause for ovulation failure. Fertilization failure in COX-2-deficient mice is presumably the result of defective oocyte maturation. Since cumulus cell-oocyte interactions are considered important for the production of fertilization-competent eggs (Eppig, 1991) and since cumulus cells surrounding the ovulated eggs exhibit perinuclear accumulation of COX-2, the absence of this isoform is most likely the cause for fertilization failure in COX-2-deficient mice.

A "two-way" interaction between the blastocyst and uterus is essential for successful implantation and subsequent decidualization. However, the molecular mechanism(s) of this interaction is not clearly understood. Numerous factors including growth factors, cytokines, homeotic genes, and PGs have been implicated in these processes (Stewart et al., 1992; Das et al., 1995; Benson et al., 1996; Chakraborty et al., 1996). Among these factors, *LIF* and *Hoxa-10* are essential, since null mutation of these genes results in defective implantation. *Ar* is also considered important because of its expression in an implantation-specific manner in the mouse uterus. The correct expression of these genes in *COX-2*(−/−) uteri on day 4 of pregnancy similar to that in wild-type uteri establishes that implantation failure in *COX-2*(−/−) females is not the result of aberrant behavior of these genes. Normal responsiveness of the *COX-2*(−/−) uteri to E_2 and P_4 is also consistent with this conclusion. COX-2 has been implicated in adhesion and invasion of tumor cells (Tsujii and DuBois, 1995; Tsujii et al., 1997). Thus, the uterine cell-specific expression of COX-2 at the implantation sites suggests that this isoform could be involved in adhesion and invasion that are major events in this process.

Among various PGs, PGE_2 has been considered as a primary candidate involved in implantation and decidualization in the rodent. It mediates its effect via interactions with G protein–coupled cell surface receptors in various cell types (Negishi et al., 1995). As described herein, the appropriate expression of PGE_2 receptor subtypes in *COX-2*(−/−) uteri suggests that uterine effects of PGE_2, if it is available, should be operative through these receptors. Thus, the failure of intraluminally infused PGE_2 to induce decidualization may indicate that this PG and its cell surface receptors may be permissive, but not essential, for this event. Further, PGE_2 effects on decidualization was claimed to be mediated via increases in intracellular cAMP (Rankin et al., 1977; Kennedy et al., 1983). Partial induction of decidualization by CTX, indeed, suggested that intracellular increases in cAMP may be involved in this process. However, failure of forskolin or dibutyryl-cAMP to mimic the same effects raises questions regarding a primary role for cAMP in decidualization as claimed previously (Rankin et al., 1977; Kennedy et al., 1983). The failure of exogenously administered PGE_2 in inducing decidualization in *COX-2*(−/−) mice reinforces a questionable role of the PGE_2-coupled adenylyl cyclase system in this process. Further, the unaltered expression of COX-1 in the uterus of either wild-type or *COX-2*(−/−) mice after application of a deciduogenic stimulus suggests that this pathway is not involved in decidualization. In contrast, rapid induction of COX-2 accompanied by massive decidualization by intraluminal infusion of oil or CTX in the wild-type uterus suggests that COX-2 is the trigger for decidualization. This is consistent with only partial decidualization by CTX in the *COX-2*(−/−) uterus; this partial decidualization by CTX could be through a mechanism not yet identified.

COX-2 is primarily localized in the nuclear envelope in various cell types (Morita et al., 1995). Therefore, the induction of COX-2, but not COX-1 (Chakraborty et al., 1996), with its distinct perinuclear localization in endometrial cells exclusively surrounding the implanting blastocyst (Figure 3C), points toward the importance of nuclear PGs in these processes. In fact, abundance of PGI_2, presumably derived by COX-2 in the absence of COX-1, at the implantation sites and expression of its newly identified nuclear receptor PPARδ in stromal cells surrounding the implanting blastocysts on day 5 of pregnancy (data not shown) suggest participation of this PG in nuclear events that could be critical for implantation. In contrast, participation of cell surface receptors for PGI_2 (IP) in this process is questionable, since IP-deficient mice have apparently normal reproductive functions (Murata et al., 1997). This reinforces the implication of nuclear receptors for PGI_2 in implantation. Thus, a limited decidual response by exogenous administration of PGI_2 agonist in *COX-2*(−/−) uteri could be due to inefficient nuclear targeting of this mediator. In a cell culture system, attempts to restore the loss of COX-2 functions by supplementation of PGs has also failed (Coffey et al., 1997), implying ineffective nuclear targeting of PGs even under in vitro conditions. Thus, if COX-2-mediated nuclear events are involved in implantation and decidualization processes, restoring these events in COX-2-deficient mice by exogenous administration of PGs in vivo could be a very demanding task. Nonetheless, participation of novel PG mediator(s) generated by COX-2 in these processes cannot be ruled out. It should be noted that implantation and decidualization processes are normal in PGF receptor–deficient mice (Sugimoto et al., 1997), suggesting that PGF2α is not crucial for these processes. In summary, the use of *COX-1* and *COX-2* null mutant mice as well as use of selective COX inhibitors establish for the first time that COX-2 is essential in several reproductive events during early pregnancy. The present investigation has clinical relevance, since selective inhibitors of inducible COX-2 have entered into phase III clinical trials as anti-inflammatory drugs. Further investigation should be directed to identify COX-2-generated PG ligands and their receptors that are involved in these events.

Experimental Procedures

Mice

The disruption of the *COX-2* gene (also called *Ptgs2*) was performed by introducing a PGK-neo cassette in place of a 1.8 kb EcoRV genomic fragment housing exon 1 and surrounding sequences (Dinchuk et al., 1995). Polymerase chain reaction (PCR) of tail DNA and blood urea nitrogen (BUN) tests (Dinchuk et al., 1995) determined the genotypes. The disruption of the *COX-1* gene (also called *Ptgs1*) was performed by replacing ~1 kb of intron 10, together with the splice junction and first 44 bp of exon 11, with the neomycin resistance gene (Langenbach et al., 1995). PCR analysis of tail DNA determined the genotypes. All of the mice used were housed in the animal care facility at the University of Kansas Medical Center.

Ovulation and Fertilization

To examine normal ovulation and fertilization, wild-type and *COX-2* (−/−) mice (C57 × 129) were bred with fertile wild-type males of the same strain. To induce superovulation, mice were injected (i.p.) with pregnant mare's serum gonadotropin (5 IU/mouse) and 48 hr later with human chorionic gonadotropin (5 IU/mouse; Sigma). They were housed with males overnight. The morning of finding a vaginal plug was designated day 1 of pregnancy. Mice were killed either day 1 or 2; oviducts were flushed with Whitten's medium (Whitten and Biggers, 1968) to recover eggs or embryos, and their morphology was examined under a microscope.

Embryo Transfer

Pseudopregnancy in wild-type or *COX-2*(−/−) recipients was induced by mating with wild-type vasectomized males. Day 4 wild-type blastocysts were transferred into the uteri of these recipients on day 4 (Paria et al., 1993). On day 5, the number of implantation sites was recorded after intravenous injections of Chicago blue B dye solution in saline. Implantation sites were demarcated as localized blue bands along the uterine horns (Paria et al., 1993). In another set of experiments, conditions of delayed implantation were induced in recipients by ovariectomy on day 4 (0900 hr) of pseudopregnancy. Delayed conditions were maintained by daily injections of P_4 (2 mg/mouse, s.c.) from days 5 to 7. On day 7, day 4 wild-type blastocysts were transferred into uteri of these mice, and implantation was initiated by an injection of E_2 (25 ng/mouse) (Paria et al., 1993). Implantation sites were examined 24 hr later. Steroids were dissolved in sesame oil (Sigma).

Decidualization

To induce decidualization, sesame oil (50 μl) was infused intraluminally in one uterine horn on day 4 of pseudopregnancy (model 1) or on a specific day of hormone treatment (model 2) as shown below; the contralateral horn served as control. Uterine weights of the infused and noninfused (control) horns were recorded and the fold increases in uterine weights were used as an index of decidualization. Decidual cell reaction was confirmed by histological examination of uterine sections. In model 1, intact pseudopregnant wild-type or *COX-2*(−/−) mice received intraluminal oil infusion on day 4 and were killed on day 8. In model 2, ovariectomized wild-type or *COX-2*(−/−) mice were treated with P_4 and E_2, which sensitize the uterus for optimal decidualization (Wordinger et al., 1986). The treatment schedule was: E_2 (100 ng) for 3 days (days 1–3), no treatment on days 4 and 5, P_4 (1 mg) + E_2 (10 ng) on days 6 and 7, and P_4 (1 mg) on days 8–11. Intraluminal oil infusion was made on day 8 and mice were killed on day 12 to record uterine weights. For reversal experiments, 1 μg of PGE_2 or carbaprostacyclin (cPGI, Cayman) in 50 μl oil was delivered intraluminally into one uterine horn. Cholera toxin (CTX, Sigma) was dissolved in PBS containing 0.1% gelatin (1 μg/ml), and 50 μl was infused intraluminally.

In Situ Hybridization

In situ hybridization was performed as described previously (Das et al., 1994). Sense or antisense ^{35}S-labeled cRNA probes were generated using appropriate polymerases from mouse-specific cDNAs to *LIF*, *Ar*, *Hoxa-10*, *COX-1*, *COX-2*, PGE_2 receptor subtypes (*EP_2*, *EP_3*, *EP_4*), and *LF* for in situ hybridization. The probes had specific activities of ~2×10^9 dpm/μg. Sections hybridized with sense probes served as negative controls.

Expression of Uterine Genes on Day 4 of Pregnancy

Sections of day 4 (0900 hr) pregnant uteri from wild-type or *COX-2* (−/−) mice were processed for in situ hybridization for *LIF*, *Hoxa-10*, *Ar*, *COX-1*, *EP_2*, *EP_3*, and *EP_4* mRNAs.

Uterine Responsiveness to E_2 and P_4

To determine whether the COX-2-deficient uteri respond to E_2 and/or P_4, wild-type or *COX-2*(−/−) mice were ovariectomized and rested for 2 weeks. They were treated with an injection of P_4 (2 mg/mouse), E_2 (100 ng/mouse), or a combination of P_4 and E_2 (Das et al., 1995). Control mice received the vehicle (0.1 ml oil). They were killed at specific times and uteri were collected for in situ hybridization.

To examine uterine cell-specific proliferation in response to E_2 and/or P_4, ovariectomized wild-type or *COX-2*(−/−) mice were given an injection of E_2 or P_4 + E_2. After 22 hr, they received an injection (i.p.) of [methyl-^{3}H]thymidine (25 μCi/0.1 ml, SA, 41 Ci/mmol; DuPont-NEN) and killed 2 hr later. Nuclear uptake of [^{3}H]thymidine was

detected in uterine sections by autoradiography (Huet-Hudson et al., 1989).

Expression of COX-1 and COX-2 in the Uterus Exposed to Deciduogenic Stimuli

To examine COX-1 or COX-2 expression in the uterus in response to deciduogenic stimuli, wild-type mice received intraluminal oil (50 μl) or CTX (50 ng/50 μl) infusion, while *COX-2*(−/−) mice received intraluminal CTX infusion on day 4 of pseudopregnancy. Uteri were collected at 2 or 8 hr later for in situ hybridization.

Immunohistochemistry

Antipeptide antibody to mouse COX-2 was produced using the peptide, NASASHSRLDDINPT, corresponding to amino acids 563–577 of the COX-2 protein, as immunogen (Chakraborty et al., 1996). Immunolocalization of COX-2 was performed in Bouin's fixed paraffin-embedded sections using a Zymed-Histostain-SP kit (Zymed) described previously (Chakraborty et al., 1996).

COX Inhibitors

Acetylsalicylic acid (Sigma), a preferential COX-1 inhibitor (Mitchell et al., 1994; Pinto et al., 1995) and DuP697 [5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene], a COX-2-selective inhibitor (Copeland et al., 1994) were dissolved in propylene glycol. Wild-type, *COX-1*(+/−) or *COX-1*(−/−) mice (129/B6) were injected with the inhibitor of either COX-1 or COX-2 on day 3 at 0800 hr and on day 4 at 0800 and 1600 hr. Control mice received the vehicle. Mice were killed on day 5 and implantation sites were recorded by the blue dye method (Paria et al., 1993).

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