Inhibition of cyclooxygenase-2 prevents inflammation-mediated preterm labor in the mouse

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current study, we tested the hypothesis that selective inhibition of COX-1 or -2 could stop inflammation-mediated PTL in the mouse by exploiting both pharmacological and genetic systems that specifically alter COX-1 or -2 activity. These trials in an animal model system provide a requisite step before considering selective COX inhibition during human pregnancy.

MATERIALS AND METHODS

Animal housing. Wild type (WT) and COX-1-deficient (KO) mice (19) were housed on a 12:12-h light-dark cycle with ad libitum access to rodent chow. All mouse protocols were in accordance with National Institutes of Health Guidelines and approved by the Animal Care and Use Committee of Washington University School of Medicine. Matings of estrous females to stud males were confirmed via copulation plug detection, and females had PTL induced with 100 µg of LPS (Escherichia coli 0111:B4; Sigma; St. Louis, MO) in phosphate-buffered saline via shallow midline, midabdominal, intraperitoneal injection at day 14.5 of gestation (~75% of term 19.5 days gestation). In our initial dose-response series 100 µg was the lowest dose of LPS that reproducibly induced PTL, without significant morbidity or mortality, in the C3H/HeN females. To determine the effect of COX inhibition, mice were pretreated with either vehicle (phosphate-buffered saline plus 1% Tween 80) or vehicle plus agent 30 min before LPS injection and redosed at 8 and 24 h after LPS. Agents were administered via gavage using dilavetted needles. Parturition phenotypes were studied in mice treated with the nonspecific COX inhibitor indomethacin at 75 and 150 µg/dose (~2.5 and 5 mg/kg, respectively), the COX-1-specific inhibitor SC-560 (Monsanto; St. Louis, MO) at 300 µg/dose (10 mg/kg), and the COX-2-specific inhibitor SC-236 (Monsanto; St. Louis, MO) at 300 µg/dose (10 mg/kg). Doses for the specific inhibitors were determined based on their previously reported IC₅₀ values for inhibition of COX-1 (29, 36) or COX-2 (29) relative to indomethacin (22).

COX-1 KO mice were generously provided by Dr. S. Morham. Production of the COX-1 KO mice has been described previously (19). The COX-1 KO mice used for our experiments were maintained on an outbred 129/Sv genetic background, with COX-1-intact littersmates of the same outbred background used as controls. Mice were 2- to 4-mo-old at the time of testing. Dose-response analysis on this outbred background demonstrated that 400 µg of LPS were required for reproducible PTL (in contrast to C3H/HeN matings) and did not result in maternal mortality (data not shown). Differences in the proportions of mice exhibiting PTL as a function of drug treatment in the C3H/HeN group and genotype in the COX-1 KO studies were assessed using a series of 2 × 2 contingency tables to calculate probabilities through use of the Fisher’s exact test (2-tailed).

Progesterone supplementation. Pregnant C3H/HeN females on day 10.5 (n = 4) of gestation were anesthetized by isoflurane inhalation and then had 15-mg progesterone pellets (Innovative Research of America; Sarasota, FL) inserted into the subcutaneous space at the nape. On day 14.5, 100 µg of LPS were administered, and the subsequent delivery phenotype was recorded. Serum progesterone levels achieved in ovariecotomized, nongravid females supplemented with these pellets were 42.5 ± 3.5 ng/ml (n = 3) as determined by radioimmunoassay (Diagnostic Products; Los Angeles, CA).

RNA analyses. Tissues harvested from euthanized mice were immediately frozen in liquid nitrogen and stored at −80°C. RNA was prepared by the guanidinium thiocyanate-cesium chloride method (6). Five (ovary) or 10 µg (uterus) of total RNA from three to six mice per treatment group were subjected to electrophoresis through 1.2% agarose-formaldehyde gels and transferred to nitrocellulose membranes. [α-32P]UTP-labeled RNA probes specific for mouse COX-1 (1,298 bp Sca I fragment in pBluescript SK II+)

RESULTS

Attenuation of PTL in the mouse by inhibition of COX-1 and -2 activity. To differentiate the relative importance of COX-1 and -2 during inflammation-induced PTL in the mouse, we initially confirmed the efficacy of indomethacin, a potent inhibitor of both isozymes, in attenuating premature delivery (34). C3H/HeN females, which had been mated with C57BL/6J
males, uniformly delivered their litters within 24 h of administration of LPS at 14.5 days of gestation (Fig. 1). In contrast, a dose-dependent reduction in PTL after LPS was seen with increasing indomethacin administration, so that at a dose of 150 µg twice daily (−10 mg·kg−1·day−1) only 20% of females delivered their litters within 24 h of LPS administration (P = 0.01 vs. LPS alone). Despite maintaining the pregnancies, most of the gravid females experienced in utero fetal deaths, with only 25% of maintained pregnancies harboring viable fetuses. Doses of indomethacin exceeding 10 mg·kg−1·day−1 resulted in maternal death, with necrotic bowel visualized at autopsy.

Term labor in mice has been shown to result from PG-induced luteolysis and progesterone withdrawal (13, 38). To determine whether the mechanism of indomethacin in attenuating LPS-induced PTL was to block progesterone withdrawal, we supplemented gravid females with sustained-release progesterone pellets to maintain serum progesterone at levels approximating those of midpregnancy. Similar to indomethacin, all progesterone-supplemented females maintained their pregnancies for more than 24 h after LPS administration (n = 4). These progesterone-supplemented females uniformly demonstrated piloerection, lethargy, diarrhea, and in utero fetal demise, suggesting that induction of PG synthesis had not been altered as a consequence of sustained serum progesterone concentrations.

COX-1 and -2 mRNA induction in uterus and ovary after LPS. Evidence for the relative contribution of each of the COX isoforms in promoting PTL was obtained by examination of induction of gene expression following LPS administration. Because luteolysis, induced by uterine- or ovarian-derived PG, is an essential component of term labor in mice (38, 40), we performed Northern blot analyses of total RNA from uterus and ovary of the LPS-treated mice. The uterus demonstrated a statistically significant threefold induction of COX-2 mRNA 2 h after administration of LPS compared with untreated controls (Fig. 2, A and B). Ovarian COX-2 mRNA in LPS-treated mice increased to a lesser extent than COX-2 mRNA in the uterus and did not achieve statistical significance (Fig. 2, A and B). In contrast, a statistically insignificant decrease in COX-1 mRNA levels was observed after LPS administration in both uterus and ovary at this time point in comparison to control mice (Fig. 2, A and C). Eight hours after LPS, COX-2 mRNA had returned to its basal level in the uterus, and COX-1 mRNA levels were unchanged compared with control mice (data not shown). As expected, administration of indomethacin with LPS did not alter the pattern of COX-1 or -2 gene expression in comparison to LPS alone (Fig. 2).

Attenuation of PTL in mouse by isoform-selective COX inhibition. Recently, pharmacological agents capable of specifically inhibiting either COX-1 or -2 in vitro and/or in vivo have been characterized. To determine the efficacy of isoform-specific inhibition in attenuating LPS-induced PTL, we analyzed the effects of specific COX-1 inhibition (SC-560) (36) and specific COX-2 inhibition (SC-236) (29) in our model system. To confirm the efficacy of SC-560 for in vivo inhibition of COX-1, we measured calcium ionophore-stimulated platelet TxB2 production, an assay that selectively evaluates COX-1 activity. WT mice treated with SC-560 showed a greater than 90% reduction in ionophore-stimulated TxB2 (Table 1). Furthermore, administration of SC-560 did not reduce serum PGE2 concentration or uterine PGF2α concentration after LPS administration in COX-1 KO mice, demonstrating COX-1 selectivity [serum PGE2 (n = 3–6 per group), SC-560 3.6 ± 0.3 ng/ml vs. vehicle 3.6 ± 0.3 mg/ml; uterine PGF2α (n = 3 per group), SC-560 4.0 ± 1.2 pg/mg tissue vs. vehicle 3.5 ± 0.2 pg/mg tissue]. Selective inhibition of COX-1 with SC-560 resulted in a moderate, statistically significant, decrease in PTL after LPS, with 58% of females delivering their litters within 24 h (P = 0.04). Of the maintained pregnancies, 40% had viable fetuses. Pregnant mice treated with SC-236, a selective COX-2 inhibitor, demonstrated a marked reduction in preterm deliveries in comparison to both mice treated with LPS alone and those treated with LPS and SC-560, such that only 8% of animals delivered litters within 24 h (Fig. 1, P < 0.001 vs. LPS, P = 0.03 vs. SC-560). Additionally, a trend toward increased fetal viability in SC-236-treated females was noted, with 73% of the maintained pregnancies harboring viable fetuses. SC-236 at the doses used to inhibit PTL did not alter platelet TxB2 production, verifying COX-2 selectivity (Table 1).

We evaluated the pattern of PG production following LPS treatment together with indomethacin, SC-560, or SC-236 administration to determine how effectively COX function had been abrogated. The results of a
one-way ANOVA indicated that the drug-treatment groups differed significantly with regard to uterine PG concentration 2 h after LPS exposure \(F(4,23) = 8.40, P < 0.0005\) (Fig. 3A). Subsequent pairwise comparisons of the uterine PGF\(_{2\alpha}\) concentrations showed the LPS alone group to be significantly higher than the control group \(F(1,23) = 9.16, P = 0.006\). Indomethacin resulted in near complete suppression of uterine PGF\(_{2\alpha}\) production after LPS administration \(F(1,23) = 22.43, P < 0.0005\), whereas LPS together with SC-236 or SC-560 resulted in PG levels similar to control mice not treated with LPS and significantly reduced in comparison to mice treated with LPS alone \(F(1,23) = 22.74, P < 0.0005\) and \(F(1,23) = 13.32, P = 0.001\) for SC-236 and SC-560 vs. LPS alone, respectively (Fig. 3A). After 8 h, uterine PGF\(_{2\alpha}\) concentrations had returned to their basal levels in mice treated with LPS alone (data not shown). The results of the data analysis pertaining to ovarian PG concentrations were similar to those found for the uterus (Fig. 3B). One-way ANOVA revealed that drug treatment groups differed significantly with respect to ovarian PGF\(_{2\alpha}\) concentration \(F(4,19) = 9.48, P < 0.0005\). Subsequent pairwise comparisons showed that the LPS alone group was significantly elevated
relative to control [F(1,19) = 17.32, P = 0.001], and
indomethacin, SC-236, and SC-560 all significantly
reduced ovarian PGF$_2$$_a$ production after LPS [F(1,19) = 33.40, P < 0.0005], [F(1,19) = 18.01, P < 0.0005], and
[F(1,19) = 22.10, P < 0.0005] vs. LPS alone for indomethacin,
SC-236, and SC-560, respectively. All P values exceed the Bonferroni-adjusted alpha level set to equal 0.01.

PTL in COX-1 KO mice. We have recently shown that
COX-1 is essential for normal term murine labor (13). To further define the role of COX-1-generated PG in
inflammation-mediated PTL, LPS was administered to
COX-1 KO mice (19). Similar to COX-1-intact control
c mice of the same genetic background, 88% of COX-1 KO mice delivered litters within 24 h of LPS administra-
tion (Fig. 4), further implicating PG generated by
COX-2 as important for inflammation-induced PTL.
Consistent with this hypothesis, Northern blot analysis
demonstrated increased COX-2 mRNA in the uterus of
COX-1 KO mice after LPS (Fig. 5A). However, the
magnitude of the induction was more variable than WT
controls of the same genetic background and thus
statistical significance was not achieved (P = 0.10).
Baseline COX-2 mRNA concentration in gravid COX-1
KO mice did not differ from WT controls. This finding is
consistent with the lack of compensation at the tran-
scriptional level of COX-2 for COX-1 in the setting of
chronic COX-1 deficiency. Evaluation of PG production
after LPS in the COX-1 KO mice revealed markedly
lower baseline and LPS-stimulated PG concentrations
in comparison to WT mice (Figs. 3 and 5B). However,
the increase in PG in the KO mice after LPS was
similar to WT mice. No significant change in uterine or
ovarian PGF$_2$$_a$ occurred in the KO mice after LPS,
suggesting that increases in other PG did not compen-
sate for the lower PGF$_2$$_a$ level (data not shown).

**DISCUSSION**

The data presented in this study demonstrate that
inhibition of PG synthesis is effective in attenuating
PTL in the mouse. Animals supplemented with exog-
igenous progesterone failed to deliver within 24 h after
LPS, lending further evidence to the notion that proges-
terone withdrawal secondary to luteolysis is the initi-
ating event for PG-induced parturition in mice. COX-2-
dependent PG production contributes significantly to
the increase in PG observed following LPS administra-
tion. Although the mechanism for term labor in mice
and humans significantly differs (4, 5), our results
suggest that effective inhibition of COX-2 may have a
central role in attenuation of PTL due to inflammation
in humans.

COX-1 activity is required for the appropriate timing
of term labor in mice, and COX-1 mRNA increases
markedly in abundance in late gestation mouse uterus
(13). In contrast, this study showed that during LPS-
induced PTL, a prominent induction of COX-2 mRNA
occurs in the uterus of WT mice, and this timing
The timing for the onset of PTL in general occurred between 8 and 24 h after administration of LPS. This delay in onset of PTL after elevation in PGF$_2\alpha$ is consistent with previous studies demonstrating a lag of up to 24 h for induction of labor after acute injection of PGF$_2\alpha$ (13) or surgical luteolysis by ovarietomy (38). The rapid induction of COX-2 is in accord with its initial characterization as an immediate early response gene in cell culture (11, 31) and has been found after LPS administration in vivo (32) as well. This increase in COX-2, taken together with the lack of significant change in COX-1 mRNA in both uterus and ovary, supports a central role for COX-2 in inflammation-induced PTL in mice. Recent studies in humans have demonstrated induction of both COX-1 and -2 (23, 24) in the extraembryonic membranes in association with PTL. In general, these studies of PTL were not limited to women with infection or other inflammatory precipitants and thus could involve either pregnancies with early onset of "term" labor (e.g., idiopathic PTL) or inflammation-mediated PTL. A variable contribution to PG production by COX-1 and -2 might depend on the underlying etiology of labor. Alternatively, COX-1 expression is very high in mouse decidua during pregnancy without LPS stimulation (13) and may have little capacity for further increases, whereas in human amnion this may not be the case. Lastly, the LPS model of PTL we utilized induces a systemic inflammatory response. Inflammation-associated PTL in humans in many cases results from initially localized inflammation due to clinical or subclinical infection within the uterus. The relative contributions of COX-1 and -2 to PG production may vary under these different circumstances.

Poor ovulation and impaired blastocyst implantation in COX-2 KO female mice precludes analysis of the pattern of delivery of gravid COX-2 KO mice after LPS-induced PTL (20). Instead, we employed selective pharmacological blockade of COX-1 or -2. We confirmed the ability of indomethacin to attenuate preterm delivery after LPS, again supporting a crucial role for PG in inflammation-related premature labor. Despite the efficacy of indomethacin in maintaining pregnancy during LPS-induced PTL, we found a high rate of fetal loss in the indomethacin-treated group. This finding suggests that the maintenance of fetal viability is not the critical determinant for attenuation of PTL by NSAIDs in mice. In WT mice, administration of the selective COX-2 inhibitor SC-236 with LPS markedly reduced the rate of preterm delivery to a level comparable to that of nonspecific COX blockade by indomethacin. SC-236...
dramatically blunted the increase in PG found 2 h after LPS, a time corresponding to the elevation seen in COX-2 mRNA. The residual uterine PGF₂α production during SC-236 treatment of WT mice is consistent with basal COX-1-generated PG because PGF₂α is nearly undetectable in COX-1 KO mice at this time in gestation (13). Specific COX-1 blockade by SC-560 proved as effective as COX-2 blockade in decreasing LPS-stimulated uterine and ovarian PG production. Inasmuch as basal PG production in the mouse uterus is predominantly attributable to COX-1, and LPS causes a twofold rise in uterine PG, we propose that specific COX-1 inhibition may reduce PTL by inhibiting basal PG synthesis and maintaining overall LPS-stimulated uterine and ovarian PG concentrations in the range of unmanipulated gravid mice.

Our results indicate that COX-1 inhibition yields an equivalent reduction in uterine and ovarian PG production in WT mice to that seen with SC-236 and yet is not as effective in attenuating PTL as specific COX-2 inhibition. As evidenced by our platelet TxB₂ assay and evaluation of PG production after LPS in COX-1 KO mice, SC-236 and SC-560 are indeed COX-2 and -1 selective, respectively. Thus the attenuation of uterine and ovarian PG production and subsequent PTL is not due to crossover effects of these agents on the other, nontargeted isoform. Although the primary role of PG is to directly induce luteolysis, the difference we observe in PTL with specific COX-1 vs. COX-2 inhibition may be attributable to the role of COX-2-generated PG in tissues outside the uterus and ovary. For instance, in the placenta, SC-236 and SC-560 are indeed COX-2 and -1 selective, respectively. Thus the attenuation of uterine and ovarian PG production and subsequent PTL is not due to crossover effects of these agents on the other, nontargeted isoform. In this case, the luteolytic consequences of COX-2 function in the context of chronic COX-1 deficiency. Second, the ability of low levels of PGF₂α to induce labor in COX-1 KO females suggests increased sensitivity of the COX-1 KO mouse to PG due to chronic deficiency. One mechanism that could lead to increased PGF₂α receptor sensitivity is a decrease in ligand-induced receptor desensitization by G protein-coupled receptor kinases and subsequent arrestin binding (9).

Selective inhibition of COX-2 efficiently attenuates inflammation-mediated PTL in mice, supporting the role of COX-2 in PTL. These studies are necessary before posing the use of COX-2-selective inhibitors in human PTL trials. However, a concern remains whether specific COX-2 vs. combined COX, or selective COX-1, inhibition results in decreased fetal morbidity. Our studies were designed to determine the roles of COX-1 and -2 in PTL and the ability of inhibitors to attenuate PTL, thus pregnancies were terminated at most 24 h after LPS to confirm pregnancy in treated females if no fetuses were expelled, as frequent resorptions occur after LPS treatment. Aborted fetuses were often cannibalized by their mothers, prohibiting the calculation of the ratio of viable to total fetuses in this paradigm. The number of pregnancies with viable fetuses at the time of death suggested that COX-2-specific blockade may result in less toxicity in comparison to combined COX or COX-1-specific inhibition. Future studies designed to assess long-term viability of pups treated in utero with COX-2-specific blockade are indicated. For example, both isoforms have been found in the ductus arteriosus, with variable contributions from COX-1 or -2 depending on the gestational age at analysis and species studied (7, 14). In addition, the diminished survival capacity of COX-2 KO mice (26) may indicate that COX-2 inhibition, especially on a chronic basis, could impart toxicity. Such studies of safety should be conducted before extrapolating our encouraging results on the inhibition of inflammation-mediated mouse PTL to therapeutic use of COX-2 inhibitors in human PTL.

Perspectives

The studies described in this report distinguishing the role of COX-1 in term labor with that of COX-2 in PTL in the mouse highlight the differences in the molecular events involved in normal and abnormal parturition. Recent studies in the mouse demonstrate that luteolysis and a fall in plasma progesterone, precipitated by activation of PGF₂α receptors on the ovarian corpus luteum, is the essential initiating event for term labor (13, 38). We now show that PG-induced luteolysis is also critical for inflammation-induced PTL in the mouse. In humans, the maintenance of late gestation pregnancy does not depend on continued luteal function, and term labor is not heralded by a fall...
in plasma progesterone (5). Determining the efficacy of COX-2-selective inhibition for treating the cascade of events culminating in human PTL poses the next challenge.

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