Intraperitoneal infusion of proinflammatory cytokines does not cause activation of the rat uterus during late gestation

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Intraperitoneal infusion of proinflammatory cytokines does not cause activation of the rat uterus during late gestation, Am J Physiol Endocrinol Metab 289: E658–E664, 2005. First published May 3, 2005; doi:10.1152/ajpendo.00058.2005.—Increased concentrations of IL-1β and TNF-α have been associated with parturition. However, the role of these cytokines is unknown. Before parturition, the uterus undergoes a process of activation, during which there are significant changes in expression of genes associated with increased uterine contractility, including the receptors for oxytocin (OT) and prostaglandin (PG)F2α (FP), PGH2 synthase isofrom 2 (PGHS2), the gap junction protein connexin-43 (Cx-43), and the inducible isoform of nitric oxide synthase (iNOS). To determine whether IL-1β or TNF-α was part of the causal mechanism for increased uterine contractions, we placed osmotic pumps infusing IL-1β or TNF-α into the peritoneal cavity of late pregnant rats (gestation day 19) and measured the effects on uterine contractility and on the uterine concentrations of mRNA for the contraction-associated genes 24 h later. Maternal serum concentrations of IL-1β and TNF-α were increased significantly. By day 21, the control animals had significant increases (P ≤ 0.05) in mRNA for OT, FP, PGHS2, and Cx-43, a decrease (P ≤ 0.05) in iNOS, and an increase (P ≤ 0.05) in uterine sensitivity and responsiveness to OT. Infusion of IL-1β or TNF-α had no effect on uterine contractility or on expression of the activation-associated genes. We conclude that intraperitoneal infusion of IL-1β or TNF-α resulting in significantly increased maternal serum cytokine levels does not cause uterine activation. The role of proinflammatory cytokines in the mechanism of parturition remains unclear.

interleukin-1β; tumor necrosis factor-α; contraction-associated proteins; uterine contractility; parturition

PRETERM BIRTH IS ASSOCIATED with ~75% of infant death and long-term disability in children. There is very poor understanding of the physiological mechanisms that regulate normal birth or the pathophysiological processes that lead to preterm birth. Before labor onset, a sequence of events, termed uterine activation, transforms the uterus from a quiescent, relatively nonresponsive muscle to a sensitive muscular organ characterized by powerful synchronous contractions that are capable of expelling the fetus. During uterine activation, the uterine lining (decidua) and muscular layer (myometrium) acquire the ability to respond to contractile stimulants such as oxytocin (OT) and prostaglandins (PG), and the myometrial cells are interconnected by increasing numbers of gap junctions that facilitate rapid cell-cell transmission of depolarizing electrical stimuli (9). Reliable markers of uterine activation include the OT receptor (OTR), the PG-synthesizing enzyme PGH2 synthase (PGHS), the receptor for PGF2α (FP), and the major gap junction protein connexin-43 (Cx-43). Increased expression of these genes, termed activation-associated genes, causes increased uterine sensitivity and responsiveness to uterotonins. There is a strong association between concentrations of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 and human parturition. In preterm labor associated with clinically evident infection, amniotic fluid concentrations of TNF-α (44), IL-1β (42), and IL-6 (40, 41, 47) are markedly increased. This pattern also occurs in normal-term labor and in preterm labor in the absence of microorganisms, although the increases in cytokines are less marked (12, 19, 32, 42, 45). Human placenta, amnion, chorion, and decidua can synthesize all three cytokines (26). Despite these strong associative data linking the proinflammatory cytokines to parturition, it remains unclear whether there is a cause-effect relationship between the cytokines and parturition.

The current study was undertaken, using an animal model, to explore a causal association between IL-1β or TNF-α and uterine activation. For in vivo experiments, we chose the Sprague-Dawley rat model because it is well characterized and the uterine changes at the end of pregnancy are similar to those of the human (14). Normal parturition occurs on the afternoon of day 21 or on the morning of day 22. Uterine activation occurs ~24 h before delivery. We hypothesized that insertion of osmotic minipumps infusing IL-1β or TNF-α into the peritoneal cavity on gestation day 19 would advance uterine activation by 24 h, such that there would be measurable changes in expression of the activation-associated genes on day 20. Additionally, we hypothesized that the uterine tissues from the cytokine-treated animals would demonstrate increased contractility, as assessed using uterine myographic techniques to determine uterine sensitivity and responsiveness to OT in vitro.

METHODS

Animals. The University of Alberta Health Sciences Animal Policy and Welfare Committee approved all animal protocols, and the experiments were conducted in accordance with the American Physiological Society’s Guiding Principles in the Care and Use of Animals. Thirty-three virgin Sprague-Dawley rats weighing ~250 g were time mated in the facilities of the University of Alberta Health Sciences Laboratory Animal Services. The pregnant animals in this colony delivered on the afternoon of day 21 or the morning of day 22. The morning of discovery of the semen plug is considered day 0. The animals were divided into five groups, including two time control groups on days 20 (before activation, n = 6) and 21 of gestation (after activation, n = 8), plus three experimental groups that received intraperitoneal osmotic pumps filled with placebo (saline, n = 6), IL-1β (10 μg, n = 8), or TNF-α (10 μg, n = 5). The intraperitoneal pumps were inserted under light inhalational anesthesia (isoflurane;
Bimeda-MTC, Cambridge, ON, Canada) through a small subhymenial incision on day 19. The Alzet model 2001D osmotic pumps (designed to deliver their contents over 24 h) were obtained from Durect (Cupertino, CA). Rats in the treatment groups were killed by heart puncture and bleeding under inhalational anesthesia. The time control animals did not receive osmotic pumps and were killed in a similar fashion to the treated animals. Five of the day 21 control animals were killed at 0900 before any signs of labor. The remaining three were killed in active labor, after delivery of at least one pup. Recombinant cytokines were obtained from Biosource International/Medicorp (Montreal, QC, Canada). Blood samples were obtained from each animal at the time of euthanasia and immediately centrifuged, and the serum was stored at −80°C until analyses. Each pump was checked to ensure that it had emptied its contents. After removal of the pups, one horn of the uterus was frozen and stored at −80°C until subsequent biochemical assessment. From the other horn, full-thickness strips of uterine wall were collected for the myographic studies.

Uterine myography. The muscle bath preparation was established on the basis of published methodology (31). Tissues from pregnant rats, gestation day 20 or 21, were obtained as described in METHODS. Uterine horns were removed immediately and incised longitudinally. No attempt was made to separate circular from longitudinal muscle or to remove attached endometrium. Uterine tissues were freed from all fetuses, membranes, and placental tissues. Longitudinal muscle strips ~8 mm in length and 3 mm in width were excised, as we attempted to avoid implantation sites.

Muscle strips were mounted vertically in separated jacketed organ baths, each containing 10 ml Krebs buffer of the following composition (mmol/l): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 0.59 MgSO4, 25 NaHCO3, 11.7 D-glucose at pH 7.4, and constantly aerated with 95% O2-5% CO2 at 32°C. One end of each strip was anchored in the bath, and the other end was attached to a Bridge 8 force-displacement transducer that was connected to a Biopac Systems MP100 unit with AcqKnowledge software, version 3.7.2 (World Precision Instruments, Sarasota, FL). A resting tension of 1 g was applied to each strip. The resting tension was determined to develop maximum active tension in pregnant Sprague-Dawley rat myometrium (14). The muscle strips were incubated for 60–90 min to establish a stable baseline. Concentration-response curves were established for OT (0.1 nM to 100 μM added at 5-min intervals), and the EC50 concentration (as a measure of sensitivity) was calculated using nonlinear regression for a sigmoid curve (Prism, version 4.0; GraphPad Software, San Diego, CA). The curve was fitted by the least square method to determine the

Quantitative reverse transcription-polymerase chain reaction. RNA was extracted using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO). Samples were treated with amplification grade DNase I (Sigma-Aldrich). The purity of the extracted RNA was assessed by spectrophotometry and the quality assessed by electrophoresis on 1% agarose gel. The sample was diluted to 50 ng mRNA/μL. Reverse transcription was performed using the Taqman Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA). The resultant cDNA was stored at −20°C until it was ready for PCR. As a negative control, the reaction was also performed in randomly chosen samples in the absence of the reverse transcriptase.

Real-time PCR was performed in triplicate using the SYBR Green PCR Core Reagents kit (Applied Biosystems). The forward and reverse primer pairs, respectively, were as follows: OTR, CGATTGCTGGGGCTTCTT and CGCCCGCCTGGCGTTTTG; TG receptor, CTGGGGCTGAATGTGCTCTGCT and TGCGGTCTTGATCTGAGAGGTT; and cyclophylin, CATGCTGCTACGAGCGTGAAGAGCACCGACAGGC; and AATGAAAGAGCCACCCAGGACCACGC; AGGTGGAAGAGCCACCCAGGACCACGC; and AATGAAAGAGCCACCCAGGACCACGC. Each protocol was individually optimized. The blank control was one tube in which autoclaved H2O replaces the cDNA template. The fluorescence was measured at the end of each PCR cycle and plotted against the cycle number to determine the threshold cycle. After amplification, the purity of the amplified cDNA was checked by assessing a melt curve of the amplified products.

Table 1. Maternal serum concentrations of IL-1β and TNF-α

<table>
<thead>
<tr>
<th>Day 20</th>
<th>Placebo</th>
<th>IL-1β</th>
<th>TNF-α</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controla</td>
<td>Pumpb</td>
<td>Pumpb</td>
<td>Pumpb</td>
<td>Controla</td>
</tr>
<tr>
<td>Cytokine</td>
<td>(6)</td>
<td>(6)</td>
<td>(8)</td>
<td>(5)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>33.7 ± 14.3</td>
<td>56.5 ± 15.7</td>
<td>124.7 ± 28.9</td>
<td>51.7 ± 23.0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.93 ± 0.66</td>
<td>2.0 ± 1.3</td>
<td>3.0 ± 1.1</td>
<td>54.6 ± 23.6</td>
</tr>
</tbody>
</table>

Values are means ± SE in pg/ml. Numbers in parentheses indicate the number of animals. aControl animals on days 20 and 21 had no pumps inserted. bOsmotic pumps were inserted on day 19, and animals were killed 24 h later. cANNOVA P < 0.05. Concentrations after IL-1β pump insertion were significantly greater than for the day 20 controls (Tukey post hoc test). dANNOVA P < 0.01. Concentrations after TNF-α pump insertion were significantly greater than for all other groups (Tukey post hoc test). eOnly 1 of the 8 samples had detectable TNF-α.
was no increase in TNF-α on the day of parturition. Except for the samples from the TNF-α-infused animals, concentrations of TNF-α were below the sensitivity of the assay in more than half of the samples. Only one of eight samples in the day 21 group had detectable levels of TNF-α.

In eight animals, we were able to collect amniotic fluid samples and compare them to maternal serum samples. For both IL-1β and TNF-α, concentrations in the two compartments were not significantly different.

Expression of activation-associated genes. Uterine concentrations of mRNA for OTR, FP, PGHS2, and Cx-43 increased significantly (P < 0.05) in the control animals by day 21 of gestation (Fig. 1). The most marked increase occurred with OTR, which increased >15-fold in the animals not in labor and >25-fold in the three that had delivered at least one pup. The increases in the other markers were in the four-to-sixfold range. In contrast, the concentration of mRNA for iNOS decreased significantly on day 21 to less than half of control values (P < 0.05). There were no differences in mRNA concentrations between the prelabor and postlabor samples on day 21, except for PGHS2, which was significantly higher after labor onset (1.6 ± 0.2 vs. 8.4 ± 1.9 relative mRNA concentration units, P < 0.001 by ANOVA).

Contrary to our hypothesis, intraperitoneal infusions of IL-1β or TNF-α for 24 h beginning on day 19 had no significant effect on the uterine concentrations of mRNA for any of the measured activation-associated genes compared with either the animals that received placebo infusions or the day 20 controls that did not have pumps inserted.

In many species, including the rat, the hormone progesterone is the major factor in maintaining uterine quiescence. Its actions are mediated through the B isoform of the progesterone receptor (PR-B). A putative mechanism of action for proinflammatory cytokines on the uterus during pregnancy is to decrease the expression of PR-B relative to the expression of the dominant negative isoform PR-A (15, 29). We have measured mRNA for PR-B and for the total of all PR isoforms (predominantly PR-A plus PR-B). We did not detect any change in the ratio of PR-B to the sum of all PR isoforms at either spontaneous term labor or after intraperitoneal infusion of IL-1β or TNF-α (Fig. 2).

Uterine myographic studies. The changes in uterine contractility were in keeping with the changes in expression of the activation-associated genes. As illustrated in Fig. 3, there was no significant increase in uterine sensitivity to OT, assessed by...
Again, there was no effect of the intraperitoneal infusions of IL-1 on uterine sensitivity. There was no effect of treatment with placebo, IL-1β, or TNF-α.

The situation with OTR is less clear. As with PGHS2, the OTR gene promoter region has multiple response elements capable of binding NF-κB or C/EBPβ (24, 25). However, we (49) and others (22, 38) have noted a decrease in OTR in human uterine myocytes after treatment with IL-1β. We noted that the negative effect of IL-1β in transformed human myometrial cells was accompanied by an increase in C/EBPβ and that the inhibitory effect could be abolished by deletion of the promoter segment containing the C/EBP response elements (49). Conversely, Terzidou et al. (51) demonstrated through the use of human myometrial cells from late pregnancy in primary culture that IL-1β caused a threefold increase in mRNA for OTR (51).

Our findings of increased expression of mRNA for FP receptor and Cx-43 levels during uterine activation are consistent with the data from others in mouse (11), rat (1, 35), and human myometrium (7, 18, 50). However, there is no direct evidence linking regulation of these genes with cytokine stimulation.

It is possible that iNOS activity that produces nitric oxide represents a mechanism to maintain uterine quiescence during most of gestation. The mRNA for iNOS was significantly increased in pregnancy at day 20 compared with tissues from nonpregnant animals (from 0.03 ± 0.01 to 0.95 ± 0.20 relative units, P < 0.01). At a time when mRNA for the contraction-associated proteins was increasing, mRNA for iNOS decreased significantly. This pattern has been noted previously for iNOS protein in human (5) and rat (2, 39). However, the current data are the first to temporally match the changes in iNOS with the other factors associated with uterine activation. This supports the concept that uterine activation is not just an upregulation of stimulatory factors but a coordinated process that also down-regulates factors causing uterine quiescence. Again, these data do not support a role for proinflammatory cytokines in uterine activation. Several studies have demonstrated that IL-1β and TNF-α stimulate iNOS expression in a variety of cell types (27). To our knowledge, there are no data demonstrating that these cytokines decrease iNOS expression.

Our experiments also failed to find any change in uterine mRNA for PR isoforms during late pregnancy. We have previously reported a decrease in the ratio of mRNA for PR-B to PR-total at the time of parturition (15). Those studies used ribonuclease protection assays to measure PR mRNA, and the difference in methods may underlie the different results. Of interest, in that study we used Western analyses to demonstrate a tendency toward increasing PR-B protein with no change in PR-A protein (30). Others have also reported significant changes in PR mRNA isoform expression at the time of labor onset in women and rhesus monkeys (20, 29). To our knowledge, no one has yet demonstrated significant changes in the relative expression of proteins for the PR isoforms.

There was no change in myographic assessment of uterine sensitivity to OT between gestation days 20 and 21 despite the marked increase in mRNA for OTR. This could indicate that increased transcription has occurred but translation has not.
However, there was a significant increase in uterine responsiveness that correlates temporally with the significant changes in mRNA for the contraction-associated genes described earlier in RESULTS. Previous in vitro studies have shown a lack of direct effect of cytokines on myometrial contractility (33). Again, the failure of IL-1β or TNF-α to reproduce any of these biochemical or functional changes suggests they are not part of the physiological pathway for uterine activation.

Two important considerations in interpreting these results relate to the animal model and the dose of cytokine used. Although there is no literature regarding effects of proinflammatory cytokines on parturition in the rat, the molecular events of uterine activation appear to be a good model for the human (14). We inserted the pumps into the lower abdomen adjacent to the uterus to take advantage of any local absorption into the uterus that might occur. We considered injection into the amniotic fluid, but that would have been technically difficult and likely inappropriate in a polytocous species such as the rat.

We chose to use 10 μg of IL-1β or TNF-α intraperitoneally in these experiments because this has been shown to produce measurable biological responses, such as elevated temperature and increased serum ACTH and corticosterone levels, in Sprague-Dawley and Wistar rats (3, 52). The IL-1β and TNF-α serum concentrations we attained are similar to those achieved in the guinea pig after instillation of Escherichia coli bacteria into the amniotic cavity to induce chorioamnionitis, causing fetal brain damage (34). In pregnant women, maternal serum levels of IL-1β and TNF-α are mostly undetectable. In amniotic fluid, concentrations are low during pregnancy but are increased slightly at the time of parturition to reach concentrations that we achieved in maternal serum following treatment (32, 44). In the presence of intrauterine infection, amniotic fluid IL-1β and TNF-α concentrations were markedly elevated to 5-to-10-fold the concentrations we achieved in maternal serum (28, 44).

Several previous studies have demonstrated that in vivo administration of a potent immune stimulus (such as LPS or bacterial products) in late pregnancy can provoke parturition. However, the underlying mechanism is unclear. In mice, intraperitoneal injection of LPS or intrauterine instillation of killed E. coli bacteria induced preterm parturition accompanied by maternal serum IL-1 concentrations similar to those we obtained (16, 23). Furthermore, mice lacking functional IL-1β receptors responded the same as wild-type animals. Thus it remains unclear whether the immune stimulus works entirely in a paracrine/autocrine fashion or whether it acts indirectly, provoking a maternal systemic response. Furthermore, the role of proinflammatory cytokines in any of these mechanisms remains unclear.

Two previous reports have described the effects of recombinant cytokines on parturition. In a chronically catheterized pregnant rhesus monkey preparation, infusion of IL-1β into the amniotic fluid caused only a transient increase in uterine contractile activity despite amniotic fluid concentrations of 10 ng/ml (4, 48). In 3 of 11 such preparations, delivery occurred within 72 h. There were no placebo or vehicle control injections in that study. In mice, subcutaneous injections of 10 μg of IL-1β in late pregnancy induced parturition within 24 h, and this could be prevented by prior administration of an IL-1β antagonist (46). Serum or amniotic fluid concentrations were not measured. When the weight of those animals is taken into consideration, this dose was ~10-fold higher than the dose used in the present studies. To our knowledge, the in vivo effects of TNF-α have not previously been studied in this regard. In summary, it appears that very large doses of exogenous cytokines or potent, nonspecific immune stimuli such as LPS or bacterial products are required to induce parturition.

Our studies intentionally used full-thickness uterine tissues, both for the mRNA measurements and for the myography studies. There is a well-described paracrine network involving the maternal decidua and myometrium that may contribute to regulation of uterine-activating factors in both the decidua and myometrium (10, 17). Both are transformed during uterine activation to influence myometrial contractility (8, 12, 13, 43). We wanted to ensure that this network remained intact through our studies.

Recent data demonstrating significant upregulation of many genes associated with the immune response strongly reinforce the notion that parturition is influenced by the immune system (21). This is in keeping with the large influx of bone marrow-derived cells into the decidua just before labor onset (53). In this circumstance, the site of cytokine production would be within the wall of the pregnant uterus. Although the present data do not support a role for proinflammatory cytokines in mediating the processes of uterine activation, it must be kept in mind that the cytokines were administered via the peritoneal cavity. The potential causative role of increased cytokine production within the uterine wall in increasing uterine contractility will be difficult to ascertain. In addition, further study is needed to determine whether the pathological increases in cytokines observed with intrauterine infection might play a causative role in those cases of preterm birth associated with such pathology. It is possible, perhaps probable, that alternate immune mechanisms other than IL-1β and TNF-α production stimulated by intrauterine pathogens may underlie uterine activation. Understanding such mechanisms may greatly assist in understanding the regulation of parturition and lead to better methods to predict or prevent the occurrence of preterm birth.

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