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Intra-amniotic administration of lipopolysaccharide induces spontaneous preterm labor and birth in the absence of a body temperature change

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Abstract

Objective—Intra-amniotic infection is associated with spontaneous preterm labor. In most cases, the infection is subclinical and bacteria are detected in the amniotic cavity rather than in the chorioamniotic membranes. The aims of this study were to establish a model of intra-amniotic lipopolysaccharide (LPS)-induced preterm labor/birth that resembles the subclinical syndrome and to compare this model to two established models of LPS-induced preterm labor/birth.

Methods—Pregnant B6 mice received an intra-amniotic, intra-uterine, or intra-peritoneal injection of LPS (100 ng/amniotic sac, 15μg/25μL, and 15μg/200μL respectively) or PBS (control). Following injection, body temperature (every two hours for a 12-hour period), gestational age, and the rate of preterm labor/birth were recorded.

Results—An intra-amniotic injection of LPS resulted in preterm labor/birth [LPS 80±24.79% (8/10) vs. PBS 0% (0/8); p=0.001] without causing maternal hypothermia. Intra-peritoneal [LPS 100% (8/8) vs. PBS 0% (0/8); p<0.001)] and intra-uterine [LPS 100% (8/8) vs. PBS

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28.57±33.47% (2/7); p=0.007] injections of LPS-induced preterm labor/birth; yet, maternal hypothermia was observed.

Conclusion—Intra-amniotic injection of LPS induces preterm labor/birth in the absence of a body temperature change, which resembles the subclinical syndrome.

Keywords

Acute histologic chorioamnionitis; clinical chorioamnionitis; endotoxin; fever; funisitis; hypothermia; infection; inflammation; mouse; parturition; pregnancy; prematurity

Introduction

Preterm birth, delivery prior to the 37th week of gestation, is the leading cause of perinatal morbidity and mortality [1-4]. Approximately 70% of all preterm births are preceded by spontaneous preterm labor [5], a syndrome caused by multiple pathological processes [6]. Of all the putative causes associated with spontaneous preterm labor, only intra-amniotic infection/inflammation has been causally linked to preterm birth [7]. Most of the intra-amniotic infections are subclinical in nature; therefore, they occur in the absence of clinical chorioamnionitis (the combination of fever, maternal or fetal tachycardia, uterine tenderness, and foul-smelling amniotic fluid) [8-11].

Microbial invasion of the amniotic cavity is the result of ascending infection from the vagina and cervix [12]. It was believed that in cases of intra-amniotic infection, bacteria were diffusely located in the choriodecidual layer prior to amniotic cavity invasion. However, it is now clear that bacteria are primarily located in the amnion, which indicates that microbial invasion of the amniotic cavity is a prerequisite for subsequent invasion of the chorion and decidua [13]. This concept is supported by three facts: (1) bacteria are detected more frequently in the amniotic cavity than in the chorioamniotic membranes of patients with a positive amniotic fluid culture [12], (2) intra-amniotic inoculation of bacteria consistently causes preterm labor/birth [14-18], and (3) inoculation of the choriodecidual space with Group B Streptococcus only leads to preterm labor when large inoculations are performed and bacteria invade the amniotic cavity [16]. In addition, lipopolysaccharide or endotoxin, a component of the cell wall of Gram-negative bacteria, can be detected in the amniotic fluid [19-22], and its concentration is higher in patients with spontaneous preterm labor than in those without labor [23]. Therefore, intra-amniotic inoculation of the bacteria, or injection of a microbial product, may resemble the human disease more closely.

LPS administration induces systemic and local inflammatory responses [24-39]. Therefore, high concentrations of endotoxin (15-250 μg per mouse) have been injected into the vagina [40, 41], peritoneal cavity [24, 42-45], and uterine horns [46-55] in order to study the mechanisms whereby microbial-induced inflammation leads to spontaneous preterm labor/birth. However, it is unclear whether these models resemble the human disease since most of the intra-amniotic infections are subclinical [8-11], and bacteria are found in the amniotic cavity instead of the chorioamniotic membranes [12]. Herein, we propose the use of a new model, the ultrasound-guided intra-amniotic injection of LPS, and compare this model to the established models of intra-peritoneal and intra-uterine injections of LPS. In addition, body

temperature was monitored in order to determine whether these models resemble the subclinical syndrome of preterm labor.

Methods

Animals

C57BL/6 (B6) mice were purchased from The Jackson Laboratory in Bar Harbor, ME, USA, and bred in the animal care facility at the C.S. Mott Center for Human Growth and Development at Wayne State University, Detroit, MI, USA. All mice were kept under a circadian cycle (light:dark = 12:12h). Females, 8-12 weeks old, were mated with males of the same background. Female mice were checked daily between 8:00 a.m. and 9:00 a.m. for the appearance of a vaginal plug, which indicated 0.5 days *post coitum* (dpc). Females were then housed separately from the males, their weight was monitored, and a gain of two or more grams by 12.5 dpc confirmed pregnancy. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Wayne State University (Protocol No. A 07-03-15).

Intra-amniotic injection of lipopolysaccharide

Pregnant B6 mice were anesthetized on 16.5 dpc by inhalation of 2-3% isoflurane (Aerrane, Baxter Healthcare Corporation, Deerfield, IL, USA) and 1-2 L/min of oxygen in an induction chamber. Anesthesia was maintained with a mixture of 1.5-2% isoflurane and 1.5-2 L/min of oxygen. Mice, positioned on a heating pad, were stabilized with adhesive tape. Fur removal from the abdomen and thorax was achieved by applying Nair cream (Church & Dwight Co., Inc., Ewing, NJ, USA) to those areas. Body temperature was maintained in the range of 37±1°C and detected with a rectal probe (VisualSonics, Toronto, Ontario, Canada). Respiratory and heart rates were monitored by electrodes embedded in the heating pad. An ultrasound probe was fixed and mobilized with a mechanical holder, and the transducer was slowly moved toward the abdomen. Ultrasound-guided intra-amniotic injection of lipopolysaccharide (LPS; *Escherichia coli O111:B4*; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 50ng (n=3) or 100ng (n=10) dissolved in 25 μL of sterile 1× phosphate-buffered saline (PBS; Fisher Scientific Bioreagents, Fair Lawn, NJ, USA) was performed in each amniotic sac using a 30-gauge needle (BD PrecisionGlide Needle, Becton Dickinson, Franklin Lakes, NJ, USA; Figure 1A). Controls were injected with 25 μL of PBS (n=8). The syringe was stabilized by a mechanical holder (VisualSonics Inc). Following ultrasound, mice were placed under a heat lamp for recovery, which occurred 10-20 min after heating. After recovery, body temperature was taken using a rectal probe and mice were monitored via video recording using an infrared camera (Sony Corporation, Tokyo, Japan) in order to determine gestational age and the rate of preterm labor/birth.

Intra-peritoneal injection of lipopolysaccharide

Pregnant B6 mice were intraperitoneally injected on 16.5 dpc with 15 μg of LPS (n=8) in 200 μL of PBS using a 26-gauge needle (Figure 1D). Controls were injected with 200 μL of PBS (n=8). This dose of LPS causes 100% of preterm labor/birth [45]. Following injection, body temperature was taken using a rectal probe and mice were monitored via video

recording using an infrared camera in order to determine gestational age and the rate of preterm labor/birth.

Intra-uterine injection of lipopolysaccharide

On 16.5 dpc, a mini-laparotomy procedure was performed as previously described [48, 55, 56]. Mice received an intra-uterine injection of 15 μg of LPS (n=8) in 25 μL of PBS using a 26-gauge needle (Figure 1G). Controls were injected with 25 μL of PBS (n=7). This dose of LPS consistently causes preterm labor/birth [55]. Following injection, body temperature was taken using a rectal probe and mice were monitored via video recording using an infrared camera in order to determine gestational age and the rate of preterm labor/birth.

Outcome Variables

Gestational age was defined as the time elapsed from the detection of the vaginal plug (0.5 dpc) through the delivery of the first pup. Preterm labor/birth was defined as delivery occurring before 18.0 dpc, and its rate was represented by the percentage of females delivering preterm among the total number of mice injected. Body temperature was taken every two hours, for a 12-hour period, following injection of LPS or PBS. In PBS-injected mice that delivered at term, body temperature was also determined every two hours for a 12-hour period prior to term labor/birth.

Statistical analysis

Statistical analyses were performed using SPSS, Version 19.0 (IBM Corporation, Armonk, NY, USA). Mann-Whitney U tests were used to analyze differences between the groups for gestational age and body temperatures, and a Fisher's exact test for the rate of preterm labor/birth. A p value of ≤0.05 was considered statistically significant.

Results

An intra-amniotic injection of 100ng of LPS per amniotic sac induced a high frequency of preterm labor/birth, whereas an intra-amniotic injection of PBS did not cause prematurity [LPS 80±24.79% (8/10) vs. PBS 0% (0/8); p=0.001; Figure 1B]. An intra-amniotic injection of 50 ng of LPS per amniotic sac did not cause preterm labor/birth (data not shown). Mice that received an intra-amniotic injection of LPS had a shorter gestational age than those that received PBS [LPS 17.35 dpc (IQR = 17.30-18.22 dpc) vs. PBS 19.19 dpc (IQR =19.12 vs. 19.38 dpc); p=0.021; Figure 1C].

An intra-peritoneal injection of 15 μg of LPS caused preterm labor/birth in all cases; yet, an intra-peritoneal injection of PBS did not have such an effect [LPS 100% (8/8) vs. PBS 0% (0/8); p<0.001; Figure 1E]. Mice intraperitoneally injected with LPS had a shorter gestational age than those injected with PBS [LPS 17.22 dpc (IQR = 17.18-17.27 dpc) vs. PBS 19.22 dpc (IQR =19.09 vs. 19.78 dpc); p<0.001; Figure 1F].

The rate of preterm labor/birth was higher after an intra-uterine injection of 15 μg of LPS than the rate following an intra-uterine injection of PBS [LPS 100% (8/8) vs. PBS 28.57±33.47% (2/7); p=0.007; Figure 1H]. Mice that received an intra-uterine injection of

LPS had a shorter gestational age than those that received PBS [LPS 17.17 dpc (IQR = 17.10-17.36 dpc) vs. PBS 19.09 dpc (IQR =17.57 vs. 19.33 dpc); p=0.001; Figure 1I].

Mice that received an intra-amniotic injection of 100ng of LPS per amniotic sac did not have a dramatic drop in body temperature (Figure 2A). Although body temperature was slightly reduced at 8 hours and at 10 hours post-intra-amniotic LPS injection, it was restored to control levels prior to preterm labor/birth (12 hours post-intra-amniotic injection with LPS; Figure 2A). Both intra-peritoneal and intra-uterine injections of LPS led to maternal hypothermia. Consequently, mice that received an intra-peritoneal or intra-uterine injection of LPS had a lower body temperature than the control groups (PBS-injected mice on 16.5 dpc and prior to term labor/birth) ($p<0.01$ in all cases; Figures 2B and 2C). In addition, mice that received an intra-uterine injection of PBS had a lower body temperature just after injection (2-8 hours post-injection on 16.5 dpc) than prior to term labor/birth (Figure 2B; $p<0.01$).

Discussion

We report a mouse model of intra-amniotic inflammation, in which an ultrasound-guided intra-amniotic injection of LPS results in preterm labor/birth without causing aberrations in body temperature. In contrast, an intra-peritoneal or intra-uterine injection of LPS induces preterm labor/birth; yet, maternal hypothermia was observed in these models.

LPS administration induces fever in humans, but not in mice [57]. In contrast, LPS administration to mice [57, 58] and rats [59, 60] causes hypothermia, which is likely due to the rapid progression of the disease and the large surface-area-to-volume ratio of these animals [61]. However, the inflammatory responses in these species are comparable [57]. For example, both mice and humans exhibited lymphopenia and increased cytokine (tumor necrosis factor-α and interleukin-6) and chemokine (CXCL1 and CXCL8 for humans and CXCL1 and CXCL2 for mice) concentrations in plasma upon LPS administration [57]. Nonetheless, it is noteworthy to mention that the dose required to produce similar inflammatory responses is higher in mice than in humans [57]. This is likely the reason why the intra-amniotic injection of 50 ng of LPS, similar to endotoxin concentrations found in the amniotic fluid of patients with intra-amniotic infection and preterm labor [23], failed to induce preterm labor and birth in mice. Yet, a double-dose of LPS (100 ng) induced preterm labor and birth.

The intra-peritoneal injection of LPS in late gestation is considered a preterm labor/birth model of systemic inflammation [62]. Therefore, this model is frequently used to investigate the mechanisms implicated in spontaneous preterm labor associated with maternal inflammatory responses, such as pyelonephritis. Acute pyelonephritis is a common complication of pregnancy [63-68], which can lead to preterm delivery [67- 74] through activation of the systemic immune response [75-85]. Accordingly, we found that the intra-peritoneal injection of LPS rapidly reduced body temperature, a sign of acute systemic inflammation in mice [57], and resulted in preterm labor/birth. However, this model of systemic inflammation may not adequately explain most preterm labors, which occur without signs of systemic inflammation [8-11].

The intra-uterine injection of LPS is commonly used as a model of local inflammation-induced preterm labor/birth [46-55]. This model mimics the human disease more adequately than the intra-peritoneal model, since microbial invasion of the uterine cavity can lead to intra-amniotic infection and preterm labor [16]. However, the intra-uterine injection of LPS requires a surgical procedure (i.e. mini-laparotomy), which itself can induce preterm labor/birth itself. Previous studies have demonstrated that sham surgeries induce neutrophil infiltration into myometrial tissues [55, 86]. Herein, the control mice that received an intra-uterine injection of PBS had hypothermia post-surgery. Further, mice that had an intra-uterine injection of LPS had a more dramatic drop in body temperature than those intraperitoneally injected with LPS. Together, these data indicate that the intra-uterine model induces local (uterus) and systemic inflammatory responses, suggesting that this model does not resemble the clinical conditions related to spontaneous preterm labor. In addition, the effects of the surgical procedure alone may confound the results and interfere with data interpretation.

Recently, we and others have shown that ultrasound-guided injections represent a new strategy to investigate the local effects of inflammatory stimuli during pregnancy [87, 88]. For example, the ultrasound-guided intra-uterine injection of LPS induces preterm labor/birth by upregulating the mRNA expression of inflammatory genes in the myometrium, chorioallantoic membranes, and placenta by increasing the infiltration of neutrophils into decidual tissues [87]. Importantly, the ultrasound-guided intra-uterine injection of PBS had no effect on the timing of parturition and did not induce neutrophil infiltration into the myometrium, suggesting that this procedure minimizes the local inflammatory response and can be used as an alternative model for studying intra-uterine infection during pregnancy [87].

The proposed intra-amniotic model resembles the human disease (preterm labor with intra-amniotic infection) more closely than the intra-peritoneal and intra-uterine models since: 1) a low dose of LPS (100 ng) was injected, mimicking the amniotic fluid concentrations of endotoxin found in women with spontaneous preterm labor [23]; and 2) the intra-amniotic injection of LPS did not cause a maternal systemic acute response, as assessed by body temperature, which is consistent with the fact that most of the intra-amniotic infections in women with spontaneous preterm labor occur in the absence of a temperature change [89, 90].

The majority (80%) of mice intra-amniotically injected with LPS delivered preterm; however, some of them (20%) delivered at term. This was expected since 87.5% of women with intra-amniotic inflammation delivered preterm and the rest delivered at term [91]. For those mice that received an intra-amniotic injection of LPS and delivered at term, it is likely that the inflammatory process induced by this endotoxin was not enough to initiate the pathway of preterm parturition. However, the adverse effects of LPS were observed since both term and preterm pups from LPS-injected mothers died shortly after delivery (data not shown).

In summary, the findings herein provide evidence that intra-amniotic injection of an endotoxin, which is able to initiate a local inflammatory response in the amniotic cavity,

induces preterm parturition in the absence of a change in body temperature. Further research is needed in order to investigate the mechanisms whereby LPS in the amniotic cavity induces spontaneous preterm labor/birth.

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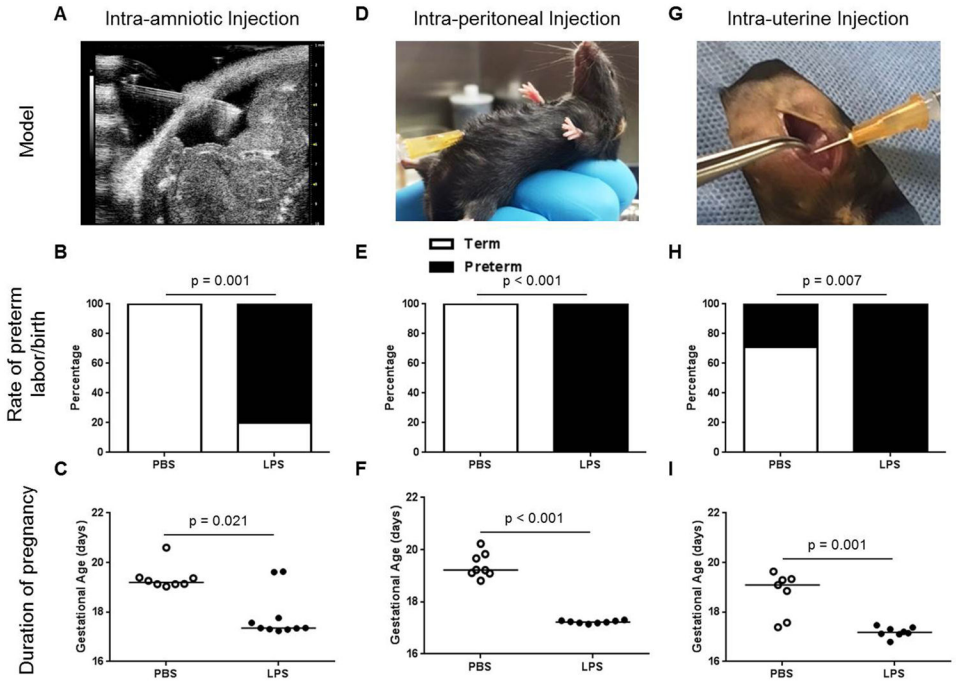

Figure 1.

Animal models of lipopolysaccharide-induced preterm labor/birth. Pregnant mice received an (A) intra-amniotic [100ng/25μL of LPS/amniotic sac (n=10) or 25μL of PBS (control, n=8)], (D) intra-peritoneal [15μg/200μL of LPS (n=8) or 200μL of PBS (control, n=8)], or (G) intra-uterine [15μg/25μL of LPS (n=8) or 25μL of PBS (control, n=7)] injection on 16.5 days *post-coitum* (dpc). Mice were video-monitored until delivery to determine the rate of preterm birth (B, E, H; data shown as stacked bars including categorical variables) and gestational age (C, F, I; data shown as scatter plots, median).

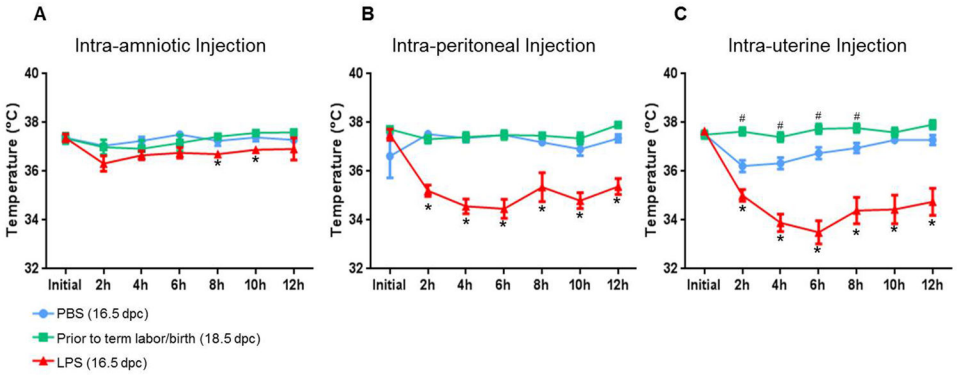

Figure 2.

Body temperatures in animal models of lipopolysaccharide-induced preterm labor/birth. Body temperatures following an (A) intra-amniotic, (B) intra-peritoneal, or (C) intra-uterine injection of LPS (red line, triangles) or PBS (control, blue line, circles) on 16.5 days *post-coitum* (dpc). Body temperatures prior to term labor/birth in mice injected with PBS (green line, squares). Data are shown as mean ± SEM; n= 7–10 each. *p<0.01 for LPS (16.5 dpc; red line, triangles) versus PBS (16.5 dpc; blue line, circles) and prior to term labor/birth (green line, squares), and #p<0.01 for PBS (16.5 dpc; blue line, circles) versus prior to term labor/birth (green line, squares).