Background: The incidence of patent ductus arteriosus is known to be higher in premature neonates with infection than in those without infection. However, the detailed mechanism has not been investigated.

Methods and Results: Lipopolysaccharide (LPS; 100 μg/kg) was injected into timed-pregnant Wistar rats on day 18 and 19 of pregnancy. The fetuses were delivered by cesarean section on gestational day 21. Using a rapid whole-body freezing method, it was found that closure of the ductus arteriosus (DA) was significantly delayed in neonates from LPS-injected rats after birth. Histological analysis demonstrated that there was no difference in vascular remodeling of the DA. Quantitative reverse transcriptase-polymerase chain reaction analysis showed that there was no difference in cyclooxygenase 2 and prostaglandin receptor, EP4, mRNA expression in the DA from LPS-injected rats. Moreover, the NOS inhibitor, Nω-Nitro-L-arginine methyl ester hydrochloride, significantly prevented the delayed closure of the DA after birth in neonates from LPS-injected rats.

Conclusions: The present study demonstrated that LPS-mediated infection delayed closure of the rat DA without apparent histological changes. iNOS, but not prostaglandin E₂, may play a primary role in delayed functional closure of the DA. (Circ J 2016; 80: 703–711)

Key Words: Ductus arteriosus; Infection; Lipopolysaccharide; Nitric oxide

The ductus arteriosus (DA) is a fetal bypass vessel between the aorta and the pulmonary artery. Patent DA (PDA) is considered a precursor to mortality and morbidity for especially premature neonates. Every third preterm infant with a birth weight (BW) of 501–1,500 g (very low BW infant) can be expected to have a persistent PDA. It has been known that 60–70% of preterm infants of less than 28 weeks’ gestation receive medical or surgical therapy for PDA to prevent respiratory decompensation, heart failure, intraventricular hemorrhage, brain injury, chronic lung disease/bronchopulmonary dysplasia, necrotizing enterocolitis and death. Importantly, the incidence of PDA is known to be even higher in premature neonates with infection than in those without infection. PDA was found in 35.3% of neonates in a tertiary care center who had late-onset sepsis (occurring after 3 days of age) proven by blood culture. The combined complications of PDA and infection worsen the prognosis of neonates. Duration of ventilator support and hospital stay were longer in neonates with sepsis in the presence of PDA than those in the absence of PDA. The requirement for additional prescription indomethacin or surgical treatment resulted in an increase in low BW preterm infants when they had a perinatal infection. Cytokine production by infection may cause PDA because tumor necrosis factor α (TNFα) is known to be increased in neonates with both infection and PDA. However, to the best of our knowledge, the mechanism of why infection induces PDA has not been further investigated.

To address this question, it is important to establish an animal model of infection-mediated PDA. Although we and others have previously reported several PDA models such as prostaglandin E₂ (PGE₂)-receptor EP4 knockout mice and Brown-Norway rats, most of them are congenital types of PDA, but not an infection-induced PDA. Lipopolysaccharide (LPS) has been widely used as an infection model in fetus and neonate. LPS is the major component of the outer membrane of Gram-negative bacteria and it triggers an inflamma-
tory cascade that results in septic shock. Bustamante et al. demonstrated that maternal administration of LPS dilated the fetal DA. Therefore, it is intriguing to elucidate the effect of LPS on the patency of the DA regarding both functional (vasoconstriction) and anatomical (vascular remodeling) closure. Functional closure of the DA is promoted by several factors such as an increase in arterial oxygen tension, a decrease in circulating PGE₂, and a decrease in nitric oxide (NO) in the luminal endothelium. The DA also exhibits a characteristic structure such as intimal cushion formation (ICF) during development, especially during a perinatal period. We have demonstrated that chronic activation of prostaglandin receptor, EP₄, promotes hyaluronic acid (HA)-mediated ICF in the DA. HA is produced by HA synthase 2 (HAS2) and HA promotes the migration of ductus smooth muscle cells. Therefore, the effect of infection on functional and/or anatomical closure of the DA needs to be elucidated. We investigated the mechanism of infection-induced PDA using maternal LPS administration in the present study.

Methods

Animals
Timed-pregnant Wistar rats were purchased from Japan SLC, Inc (Shizuoka, Japan). All animals were cared for in compliance with the guidelines of the American Physiological Society. The experiments were approved by the Ethics Committee on Animal Experiments of The Jikei University.

LPS-Injected Rat Model
In this study, we used LPS to create a model of chorioamnionitis as a severe infection. Pregnant Wistar rats were anesthetized with isoflurane and intraperitoneally injected with LPS (100 μg/kg) on gestational days 18 and 19. For the control group, the same volume of PBS was injected into pregnant rats in the same manner. Rat fetuses were delivered by cesarean section (CS) on embryonic day 21. LPS from Escherichia coli 055: B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Rapid Whole-Body Freezing Method
To study the in situ morphology and inner diameter of the DA and the main pulmonary artery (MPA), a rapid whole-body freezing method was used, as previously described, with some modifications. The fetuses from LPS- or PBS-injected rats were delivered by CS on embryonic day 21. The neonates were placed into a warm incubator for 30 or 60 min and were then rapidly frozen in liquid nitrogen. The frozen neonates were cut on a freezing microtome in the frontal plane, and the inner diameters of the DA and the MPA were measured under a microscope. We evaluated the patency as the ratio of the DA diameter to the MPA diameter.

Histological Analysis
Tissues from the placenta, DA and aorta were obtained from LPS-injected rat models. Paraffin-embedded blocks containing these tissues were cut into 3μm-thick sections and placed on glass slides. To observe polymorphonuclear leukocyte infiltration, the tissue sections were stained with hematoxylin and eosin (HE). To determine the ICF boundary line, tissue sections of the DA and aorta were stained with Elastica van Gieson stain.

Immunohistochemistry
HA staining was performed by using sections of the DA and the aorta, as previously described. Briefly, the specimens were deparaffinized, rehydrated, incubated for 30 min in a peroxidase blocking regent to inactivate endogenous peroxidases, and incubated for 30 min with 1% bovine serum albumin with PBS-T to avoid non-specific staining. Tissue sections were incubated with biotinylated HA-binding protein (United States Biological, Salem, MA: 8μg/ml) at room temperature for 2h. The slides were sequentially incubated for 30 min with a biotinylated secondary antibody, incubated for 30 min with VECTASTAIN Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA) and incubated for 5 min with DAB Peroxidase Substrate (Vector Laboratories). The sections were rinsed and stained with hematoxylin and mounted.

Quantitative Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Analysis
Total RNA was isolated from pooled DA and aorta tissue from neonates from LPS- or PBS-injected pregnant rats at birth using TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). cDNA was generated using PrimeScript™ RT Master Mix (Perfect Real Time, Takara, Otsu, Japan). RT-PCR analysis was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus), Bulk (Takara). After initial denaturation at 95°C for 30s, the PCR cycle consisted of denaturation at 95°C for 5s and annealing and elongation for 60°C for 45s; 45 cycles was performed. The sequences of primers for TNFa, interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), endothelial NO synthase (eNOS), cyclooxygenase 2 (COX2), EP₄, HAS2 and Toll-like receptor 4 (TLR4) are listed in Table S1. The abundance of each gene was determined relative to an internal control using 18 s ribosomal RNA. The sequences of the 18 s ribosomal RNA primer are listed on Table S1. For each RT-PCR experiment, which included a RT negative control, we confirmed that there was no artificial amplification in any reaction.

NO Synthase Inhibition
To investigate the effect of a NOS inhibitor, we injected No-Nitro-L-arginine methyl ester hydrochloride (L-NAME) or saline into the fetuses from LPS-injected maternal rats intraperitoneally through the uterine wall during a CS. The neonates were placed into a warm incubator for 30 min and were rapidly frozen in liquid nitrogen as explained above. L-NAME was purchased from Sigma-Aldrich.

Statistical Analysis
All data are presented as the mean ± standard error of the mean (SEM) of independent experiments. Statistical analyses were performed using the Mann-Whitney test for the 2 groups that were not normally distributed. A P value of less than 0.05 was considered statistically significant.

Results

Maternal Administration of LPS Delayed Closure of the DA in Rat Neonates
To examine the effect of inflammation on DA closure, we observed the DA after birth using the rapid whole-body freezing method. We injected LPS (100 μg/kg) into 25 pregnant rats at embryonic day 18 and 19. Among them, 23 pregnant rats could survive until a CS on embryonic day 21. All 19 PBS-injected pregnant rats survived. Closure of the DA was significantly delayed in neonates from LPS-injected rats when they were observed 30 and 60 min after birth (Figures 1A–F). In neonates from PBS-injected control rats, the DA diameter was decreased up to 20% of the MPA within 30 min after birth and
LPS Causes PDA by iNOS But Not PGE₂

ICF Developed Normally in the DA of Fetuses From LPS-Injected Rats

ICF is an essential remodeling process for postnatal closure of the DA. We found that ICF normally developed in the DA of fetuses from both LPS- and PBS-injected rats.
DA and aorta, although it did not reach a statistical significance (Figure 4G; n=6). We examined the expression levels of voltage-gated potassium channels (Kv)1.5 and Kv2.1 mRNAs, because they are known to play important roles in closure of DA. There were no significant change in LPS-stimulated DA compared with PBS-injected DA (Figure S1).

L-NAME Inhibited LPS-Induced Patent DA
When the NOS inhibitor, L-NAME, was injected into the fetuses from LPS-injected maternal rats intraperitoneally through the uterine wall during a CS, the lumen of the DA was significantly decreased up to 17% of the MPA 30 min after L-NAME injection when compared with the lumen of the DA of saline-injected neonates, which was 31% of the MPA (Figures 5A–C).

Maternal Administration of Lipopolysaccharide Caused Chorioamnionitis
Macroscopically, the placentas from PBS-injected pregnant rats were lucent and clear (Figure 6A), but the placentas from LPS-injected pregnant rats were dull and dirty (Figure 6B). Histologically, we observed few polymorphonuclear leukocytes on the placentas from PBS-injected pregnant rats (Figures 6C,D), whereas inflammation, indicated by infiltration of polymorphonuclear leukocytes, was extended into the chorion of placentas from LPS-stimulated pregnant rats (Figures 6E,F; n=9–14).

Discussion
Patent DA is considered a precursor to mortality and morbid-
LPS Causes PDA by iNOS But Not PGE2

In the present study, we demonstrated that maternal administration of LPS delayed closure of the DA by impairing functional closure through TNFα and iNOS induction. However, the effect of LPS on PDA is controversial. Vucovich et al reported that PDA with infection was caused by aminoglycoside antibiotics, but not by LPS. In the in vivo experiment by Vucovich et al, mice but not rats were treated, and newborn term gestation mice were injected with LPS at 30 min of age and then the DA was assessed after a 4-h period of observation. Therefore, the duration of LPS exposure was much shorter in their study than our and Bustamante’s experiments, where chronic inflammatory responses were observed. Thus, we suggest that maternal LPS administration is a suitable method to investigate the mechanism of PDA with infection.

The DA from LPS-injected rats did not show any significant histological changes. Closure of the DA depends on 2 mechanisms: functional vasoconstriction and anatomical vascular remodeling. Maternal rubella infection is known to cause PDA in the fetus that exhibits abnormal anatomical remodeling such as less ICF. Because we did not find any significant histological changes in the DA of fetuses from LPS-injected rats, the delayed closure of the DA is mainly a result of impaired functional closure of the DA.

Although there were no histological changes in the DA of the fetuses from LPS-injected rats, we found chorioamnionitis in the placenta from LPS-injected rats. Because chorions are parts of the fetus, chorioamnionitis indicates that maternal administration of LPS induced infection in the fetus. Several previous studies have demonstrated that chorioamnionitis is significantly related to PDA, whereas another study did not show a significant relationship between chorioamnionitis and PDA. Our in vivo study indicates that maternal LPS admin-

Figure 3. Hyaluronic acid production did not change between DAs stimulated by either LPS or PBS. HA production was visualized by staining for HABP. (A) The DA and aorta from fetuses stimulated by PBS. (B) The DA and aorta from fetuses stimulated by LPS. (C, D) Magnified DA of (A) and (B). (E) There was no significant change in HAS2 mRNA expression in the DA. The values are expressed as mean±SEM. P<0.05. (A–D, n=5; E, n=6). HA, hyaluronic acid; DA, ductus arteriosus; Ao, aorta; LPS, lipopolysaccharide; PBS, phosphate buffered saline; HABP, HA binding protein; HAS2, hyaluronic acid synthase 2.
production of TNFα and NO and a variety of additional inflammatory mediators. In addition, LPS produces iNOS via eNOS and TNFα, which contributes to LPS-induced hypotension. We initially hypothesized that LPS-induced PGE2 caused PDA. However, we found no significant differences in COX2, EP4

Figure 4. Quantitative RT-PCR shows TNFα, IL-6, iNOS, eNOS, COX2, EP4 and TLR4 mRNA expression in rat DA and Ao. (A, C) TNFα (A) and iNOS (C) mRNA expression levels in LPS-injected DA and aorta were significantly increased compared with PBS-injected DA and aorta. (B, D) IL-6 (B) and eNOS (D) mRNA expression levels in LPS-injected DA and aorta were not significantly increased compared with PBS-injected DA and aorta. (E, F) COX2 (E) and EP4 (F) mRNA levels in LPS-injected DA and aorta were not significantly increased compared with PBS-injected DA and aorta. (G) TLR4 mRNA levels in LPS-injected DA and aorta tended to increase compared with PBS-injected DA and aorta. The values are expressed as mean±SEM. *P<0.05, **P<0.05 (n=6). Quantitative RT-PCR, quantitative reverse transcriptase-polymerase chain reaction analyses; TNFα, tumor necrosis factor α; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; COX2, cyclooxygenase 2; EP4, prostaglandin receptor EP4; TLR4, Toll-like receptor 4; DA, ductus arteriosus; Ao, aorta; LPS, lipopolysaccharide; PBS, phosphate buffered saline.

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registration caused chorioamnionitis, which then induced inflammatory responses in the fetuses, resulting in PDA.

It is also important to identify the mechanism by which LPS administration impairs functional closure of the DA. LPS triggers an inflammatory cascade, resulting in septic shock in macrophages through TLR4. This response is characterized by
LPS Causes PDA by iNOS But Not PGE₂

Figure 5. Nitric oxide synthase inhibitor inhibited LPS-induced PDA. (A, B) Representative images of saline-injected neonatal rat (A) and L-NAME-injected neonatal rat (B) 30 min after L-NAME or saline injection. (C) L-NAME injection significantly inhibited LPS-induced PDA. The values are expressed as mean±SEM (C, n=15–16) *P<0.05. DA, ductus arteriosus; Ao, aorta; LPA, left pulmonary artery; LPS, lipopolysaccharide; L-NAME, Nω-Nitro-L-arginine methyl ester hydrochloride; PDA, patent DA.

Figure 6. Inflammation was observed in placentas from LPS-injected pregnant rats. (A) A representative placenta from PBS-injected pregnant rats was macroscopically clear and lucent. (B) A representative placenta from LPS-injected pregnant rats was macroscopically dull and dirty. (C, D) Hematoxylin and eosin (HE) staining of the placenta from a PBS-injected pregnant rat. (D) Magnified square from (C). Polymorphonuclear leukocytes were not observed. (E, F) HE staining of the placenta from LPS-injected pregnant rats. (F) Magnified square from (E). Polymorphonuclear leukocytes were observed. Scale bars: (A, B) 5 mm; (C–F) 100 μm. LPS, lipopolysaccharide; PBS, phosphate buffered saline.
and HAS2 mRNA expression and HABP staining in the DA of LPS- and PBS-injected rats, suggesting that the PGE2-EP4 pathway did not contribute to LPS-mediated PDA. Instead, we found that TNFα and iNOS mRNA expression was significantly increased in the maternally LPS-injected DA compared with in the maternally PBS-injected DA. Bustamante et al showed that a NO inhibitor closed the LPS-induced PDA, which is consistent with the findings of the present study. Therefore, we think that NO plays a primary role in PDA by LPS-induced infection. We showed that the NOs inhibitor, L-NAME, inhibited LPS-induced PDA in mature fetuses. It should be noted that the dilatory effect of NO on the DA is more significant in immature fetuses. 46–47 Momma and Toyono reported that NO, but not PGE2, played a major role in dilating the DA in preterm fetal rats (embryonic day 19), 48 and that the importance of NO was relatively decreased during development. 49–50 Clyman reviewed that the DA in preterm newborn produced an increased amount of NO after birth although the DA responded to PGE2 persistently. 49 This report supports the study by Momma and Toyono. 48 These findings including the present study suggest that NO inhibitors could be a therapeutic agent to prevent infection-related PDA, especially in immature fetuses. In addition to NO, it has been known that reactive oxygen species and calpain 49 are therapeutic targets for inflammation-related vascular diseases. Therefore, further investigation may be required to uncover the role of reactive oxygen species and calpain in inflammation-induced PDA.

In conclusion, maternal administration of LPS delayed closure of the DA by impairing functional closure. This impairment is likely to result from TNFα and iNOS but not the PGE2-EP4 pathway. Our present study suggests that NO inhibition is an effective alternative strategy to COX2 inhibitors for closing the DA when neonates are exposed to severe infection.

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References

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Supplementary Files

Supplementary File 1

Methods

Table S1. The sequences of primers

Figure S1. Quantitative RT-PCR shows (A) voltage-gated potassium channels (Kv) 1.5 and (B) Kv2.1 expression in the rat DA and Ao. Please find supplementary file(s): http://dx.doi.org/10.1253/circj.CJ-15-1053