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Vaginal LPS changed gene transcriptional regulation response to ischemic reperfusion and increased vulnerability of fetal brain hemorrhage

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ABSTRACT

During pregnancy, both ischemic reperfusion and bacterial agent LPS are known risk factors for fetal brain damage. However, there is a lack of evidence to explain whether vaginal LPS affects the fetus response to ischemic reperfusion. Here we reported that there was more than 2 folds higher vulnerability of fetal brain hemorrhage response to ischemic reperfusion when mother mouse was treated with vaginal LPS. As our previously reported, ischemic reperfusion induces P53-dependent fetal brain damage was based on a molecular mechanism: the transcriptional pattern was changed from HIF-1alpha-dependent to P53-dependent immediately. In the present work, only with vaginal LPS precondition, phosphorylation of activated transcriptional factor (ATF) 2 at Thr71 appeared in response to ischemic reperfusion. Moreover, this phosphorylation was completely blocked by pre-treatment with a P53 inhibitor, pifithrin-α. We concluded that vaginal LPS precondition trigged the p53-dependent phosphorylation of ATF2 in response to ischemic reperfusion, which played an important role of increasing vulnerability to hemorrhage in fetus.

1. Introduction

Fetal brain damage induces pregnancy loss or severe diseases like cerebral palsy. Ischemic reperfusion is a risk factor of fetal brain damage in the late preterm period during pregnancy. Ischemic reperfusion in pregnant women is usually caused by intrauterine asphyxia [1]. However, ~70% pregnant women have experiences on bacterial vaginosis (BV) including subclinical BV [2,3] a risk factor of preterm birth and fetal brain damage [4–6]. Expose to both ischemic reperfusion and subclinical BV during pregnancy is not rare. However, there is no answer to only a simple question "whether vaginal inflammation derived from subclinical BV has effect on fetal brain damage". In the present work, we first investigated whether vaginal bacterial lipopolysaccharide (LPS) effects fetal response to ischemic reperfusion.

Fetal brain damage varies according to the stage of brain development and the method of ischemic reperfusion or LPS induction. Animal experimental data has shown that either Ischemic reperfusion (20 min) or extra-uterine LPS (0.3 mg/kg i.p.) exposure fails to induce fetal brain damage whereas intra-uterine LPS exposure prior to birth causes a normal neuronal dopamine reduction [4,5]. Moreover, LPS given 4 h prior to Ischemic reperfusion produces infarction in brains of 7-day-old rat pups [6]. Therefore, all these imply that LPS sensitizes the immature brain to Ischemic reperfusion. However, it is still unknown whether vaginal LPS administration induces the fetal brain damage response to ischemic reperfusion.

LPS induces an inflammatory reaction via activating a wide range of signaling pathways such as the mitogen-activated protein kinases (MAPKs) and Akt pathways. MAPKs mainly include extracellular signal regulated kinase (ERK), P38 and c-Jun N-terminal kinase (JNK). Phosphorylated forms of these proteins regulate various target genes including *p53* and activate transcriptional

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factor (*Atf*) *2* [7–9]. The transcriptional activities of P53 and ATF2 are proven to be dependent on their phosphorylation [10,11]. P53-dependent cell death has known to play an important role during development and various types of brain damage such as subarachnoid hemorrhage [12–15]. As our previous report [16], response to IR, P53 was a central molecular response to immediately regulate the gene expression within 30 min after ischemic reperfusion. Moreover, current evidence shows that pretreatment with pifithrin-α (PFT-α) can reduce ischemic brain injury through p53 transcriptional activity inhibition in mice [14,15]. On the other hand, phosphorylation of ATF2 at Thr71 was recorded in adult rat brain response to various stresses such as osmotic stress and transient cerebral ischemic reperfusion [17,18].

2. Materials and methods

All animal experiments in this study were conducted in accordance to the Tohoku University guidelines for animal experimentation. The Tohoku University Committee for Safety Management of Animals approved all the experimental protocols in this study.

2.1. Mice

C57BL/6N mice were used for all experiments. Mice were purchased from CLEA Japan Inc. (Japan). Mice weighing 18–22 g (9–14 weeks) were bred overnight marking gestation day 0 (GD 0) under specific pathogen-free conditions at the Animal Research Institute of Tohoku University. Specifically for the sacrifice/euthanasia, anesthesized mice was be killed by using ketamine (Ketalar 500 mg®Daiichi-Sankyo: 100 mg/kg) and xylazine (ROMPUN INJ. SOLUTION 2%®Bayer; 10 mg/kg).

Every day, body weight and food intake of pregnant mice were measured, there were no significant changes between all groups.

2.2. Inflammation induced by LPS administration

To induce vaginal inflammation during the late gestational period, LPS administration was selected to start on GD14 in our murine model (Supplementary Fig. 1a). Either vaginal saline or amniotic LPS (10 μl of 1 mg/ml per fetus) was used as a control.

Vaginal LPS was administered as previously described [21]. Specifically, 0.3, 3 or 30 μg/vagina of LPS was administered twice on GD14 and GD16. During these dates, GD14 to GD18, that body weight is observed to increase, such as from 31.8 ± 1.2 g–37.9 g ± 0.8 g ($n = 3$ dams). During this period, there is acceleration of the fetal brain development in humans [22]. Our data has shown that 30 μg LPS using current method induced premature birth (2/5 dams). Thus, we adopted the confirmed LPS concentration of 3 μg/vagina to induce a subclinical inflammatory in vagina without premature birth. However, only a single administration of 3 μg vaginal LPS on GD14 produced no inflammatory cells in the vagina on GD18 as detected by Hematoxylin and Eosin staining, therefore a continuous administration through to GD16 was necessary to maintain local inflammation until GD18, the day of IR treatment. Double LPS administration in the vagina every two days from GD14 was performed to replicate an antenatal infective process.

2.3. Intrauterine ischemic reperfusion

In present work, ischemic reperfusion was induced on GD18 as previously described [16]. Because ischemic reperfusion in pregnant women is usually caused by intrauterine asphyxia [1], such as in the case of women in labor wherein uterine contractions cause ischemia and uterine relaxation allows reperfusion. Aseptic conditions were maintained all throughout the experiment. During the surgery, temperature was controlled at 36.5 ± 1° and humidity at 65 ± 5%.

2.4. P53 inhibitor pre-treatment

To inhibit P53 activity in the fetal brain, Pifithrin (PFT)-α (sc222176, SANTA CRUZ) was administered as previously described [16]. Specifically, a single subcutaneous (s.c.) injection was administered 3 or 6 h (described as −3 or −6 in the Supplementary information) prior to ischemic reperfusion on day 18 of gestation (GD18).

2.5. Fetal brain population

Pregnant mice were separated into six conditions: 1. No vaginal treatment ($n = 10$); 2. Vaginal saline administration (saline: $n = 10$); 3. Vaginal LPS administration (LPS: $n = 52$); 4. Only ischemic reperfusion by clipping of a unilateral uterine horn for a total of 15 min on GD18 (IR: $n = 12$); 5. Vaginal saline plus ischemic reperfusion ($n = 16$); 6. Vaginal LPS plus ischemic reperfusion ($n = 54$). Brain samples were derived from 190 fetuses from 52 mouse dams.

Fetal brain sampling was performed on GD18. In study conditions involving fetal brain ischemic reperfusion, samples were immediately collected after reperfusion and all steps of sampling were finished within 30 min.

2.6. Sections and imaging

To evaluate LPS induced inflammation, vagina and brain samples were dissected and fixed for 3 days in 15 ml of 4% paraformaldehyde (PFA) or frozen immediately using dry ice. Paraffin-embedded samples (3 μm) were analyzed by Hematoxylin and eosin (HE) staining.

To evaluate the vulnerability of fetal brain hemorrhage, microscopic examination of frozen brains samples in 10 μm sections were used to ascertain evidence with HE staining as control.

2.7. RT-PCR

Total mRNA extracted from 5 fetal brains from each treatment group were utilized. RT-PCR primers were designed by Primer3Plus software. The sequences used as primers have been provided as Supplementary Table 1.

2.8. Western blotting

We investigated protein changes within 30 min after IR in the fetal brain.

As previous report [16], for western blot analysis, tissues were flash-frozen in liquid nitrogen and stored at −80° until use.

Antibodies (1:1000) used were purchased from Cell Signaling Technology: Anti-Actin (5125), anti-TLR4 (2219), anti-phospho rylation of ERK1/2 at Thr202/Tyr204 (4376), anti-phosphorylation of p38 at Thr180/Tyr182 (9211), anti-phosphorylation of AKT at Ser473 (4060), anti-SAPK/JNK (9258), anti-phosphorylation of SAMP/JNK at Thr183/Tyr185 (4668), and anti-phosphorylation of ATF2 at Thr71 (9221).

Western blot data were analyzed using Image J software. Graphs were made and analyzed using Graphpad Prism 5 software.

2.9. ELISA

A blood sample was collected into a pre-cooled EDTA tube. For

amniotic fluid samples, those from fetuses from the same mother were mixed and collected into a 1.5 ml tube. All samples were immediately centrifuged at 2200 g for 15 min at 4 °C. The supernatants were stored at −80 °C until analysis. The mouse inflammatory cytokines multi-analyte ELISArray kit (MEM-004A, SABiosciences QIAGEN) was used to detect 12 types of pro-inflammatory cytokines in the plasma or amniotic fluid samples. The cytokines included in the array are IL1A, IL1B, IL2, IL4, IL6, IL10, IL12, IL17A, IFNγ, TNFα, G-CSF, and GM-CSF.

2.10. Fetal electrocardiography (FECG)

As a previous report [16,23,24], FECG was used to evaluate the situation of fetuses (PCT/JP2006/316386). Specifically, if necessary, FECG was continuously measured within 3 h after ischemic reperfusion.

2.11. Statistical analysis

Parameters derived were expressed in mean ± standard deviation (SD). The statistical method used was the Student's *t* test (two tailed for independent samples) for two group comparison. For multiple group comparison, statistical analysis was performed using the Kruskal–Wallis one-way ANOVA. A probability (p) of less than 0.05 was considered significant.

3. Results

3.1. Vaginal LPS may sensitize the fetal brain response to ischemic reperfusion

Fetal hemorrhage has been considered as a major etiology of cerebral palsy, specifically in the late gestation [5,19,20]. To investigate whether vaginal LPS is a risk factor for fetal brain damage when exposed to ischemic reperfusion (IR), intracerebral hemorrhage incidence was investigated. According to a grading system for fetal hemorrhage [25], most cases in the vaginal LPS plus IR group were classified as Grade I (Fig. 1d). Within 30 min post-IR, LPS administration notably increased the vulnerability of hemorrhage due to IR from 2/8 to 7/13 (Fig. 1e). This result implies that the risk of intracerebral hemorrhage is higher (25% Vs 53.8%) with vaginal LPS precondition. This is in agreement with previous studies [6,30], where Vaginal LPS also sensitized the immature brain response to IR.

3.2. Vaginal LPS induced a significant local inflammation and increased phosphorylation of JNK response to ischemic reperfusion in the fetal brain

To evaluate the inflammation induced by vaginal LPS, vagina was collected on GD18 and analyzed by HE staining (Fig. 2a). Inflammatory cells specifically appeared at the junction between the vagina and uterus in the LPS treated mice. Using fetal electrocardiograph, it has been shown that vaginal LPS did not significant change the character of heart beat rate response to 3 cycles of 5 min ischemic reperfusion [16] (Fig. 2b–h). Under vaginal LPS precondition, Ischemia reduced heart beat rate, oxygen concentration in the amniotic fluid, and phosphorylation of proteins including Akt at Thr202/Tyr204 and Erk at Ser473 (Fig. 2i, Supplementary Fig. 1a), while reperfusion rescued heart beat rate immediately. On this endpoint time, 12 types of cytokines (see material and methods), including IL-6, TNF and G-CSF were found to be negative in the plasma of mouse dams (Fig. 2k, compare with amniotic LPS induced positive cytokine release in Supplementary Fig. 1b).

On the other hand, phosphorylation of JNK1/2 at Thr183/Tyr185 was increased in the LPS plus IR group (Fig. 3a). As our previously report [16], IR inhibits most protein phosphorylation activity [1], so that increased phosphorylation of JNK1/2 was of primary focus in this study.

3.3. Quick phosphorylation of ATF2 at Thr71 needs both LPS and ischemic reperfusion

Stress-inducible transcriptional factor ATF2 is a known substrate of MAPKs and its phosphorylation is enhanced by JNK activation [9,26–28]. In mice, ATF2 is highly expressed and activated in the brain and it plays a critical role in mouse embryos [29]. Only under vaginal LPS precondition, phosphorylation of ATF2 was strongly detected response to IR (Fig. 3a). Next, we confirmed that phosphorylation of ATF2 was not caused by experimental stimuli or was not due to LPS alone. Either only ischemic reperfusion, only vaginal LPS (even 30 μg LPS in Fig. 3c; the last lane from left), vaginal saline plus ischemic reperfusion (Fig. 3a; lane 4), or only a single vaginal administration of 3 μg LPS on GD14 (Supplementary Fig. 1c), they all did not induced detectable phosphorylation of ATF2 at Thr71.

3.4. Pifithrin (PFT)-α suppressed phosphorylation of ATF2 in LPS groups

Ischemic reperfusion immediately changed transcriptional partner from HIF-1alpha-dependent to P53-dependent manner [16]. P53-dependent cell death is a major reason of fetal brain damage including hemorrhage [12, 13, 14, 15]. To understand the role of above phosphorylated ATF2, we further investigated the link between ATF2 and P53. Ischemic reperfusion phosphorylated JNK1/2 in the fetal brain was partly suppressed by pretreatment with PFT-α 3 and 6 hs (Fig. 3c). Furthermore, the phosphorylation of ATF2 at Thr71 was completely suppressed by PFT-α (Fig. 3c). This result implied that a P53-dependent phosphorylation of ATF2 at Thr71 was through the phosphorylated JNK1/2 particularly.

In addition, ischemic reperfusion reduced protein syntheisis including phosphorylation of mTOR and S6, while they were increased with vaginal LPS precondition (Supplementary Fig. 1d). In contrast, phosphorylation of S6 in the fetal brain was almost suppressed by PFT-α (Fig. 3c). This result suggested that increased protein synthesis in the LPS plus IR group was also regulated by p53.

4. Discussion

In the present work, it has been suggested that phosphorylation of ATF2 was a biomarker of fetal hemorrhage induced by vaginal LPS plus ischemic reperfusion. How does this phosphorylation of ATF2 occur in the fetal brain? An evolving relationship between ATF2, HIF-1α and P53 should be very interesting in the fetal brain. Phosphorylation of ATF2 is reported to have not only P53-independent transcriptional activity but also a functional association with P53 [29,31]. Stable levels of ATF2 regulate transcriptional activity of HIF-1α by competing with P53 [32]. There was a novel link between ATF2-P53-JNK1-HIF-1α such as it has documented in colorectal cancer [33]. Moreover, P53 inhibitor experiments suggested that phosphorylation of ATF2 at Thr71 needed P53 (Fig. 3c). We proposed that this P53-dependent phosphorylation of ATF2 at Thr71 in the LPS group may be a specific sensitive signal of fetal brain response to IR. A possible explaining is: Ischemic reperfusion induced HIF-1α-dependent to P53-dependent transcriptional regulation manner [16]. Vaginal LPS was through cell signaling pathway to regulate cytokine expression including TNF-α to sensitize fetal brain (Supplementary Fig. 1e). As well known, LPS induced inflammatory reaction has two aspects: repair and brain

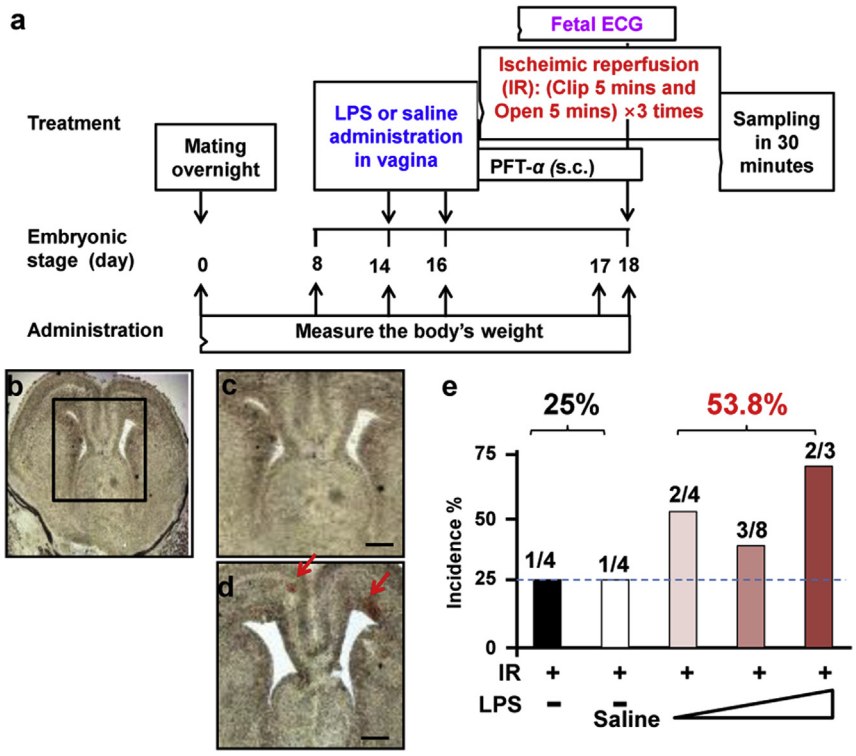

Fig. 1. Vaginal LPS might increase the vulnerability of fetal brain damage response to ischemic reperfusion. (a) The mouse model used in the present work. (b) Whole fetal brain, (c) negative hemorrhage (no treatment) and (d) positive hemorrhage (red dose). (e) Relative percent of hemorrhage incidence under indicated conditions. Numbers at the top of column mean the positive hemorrhage/total number of fetal brain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

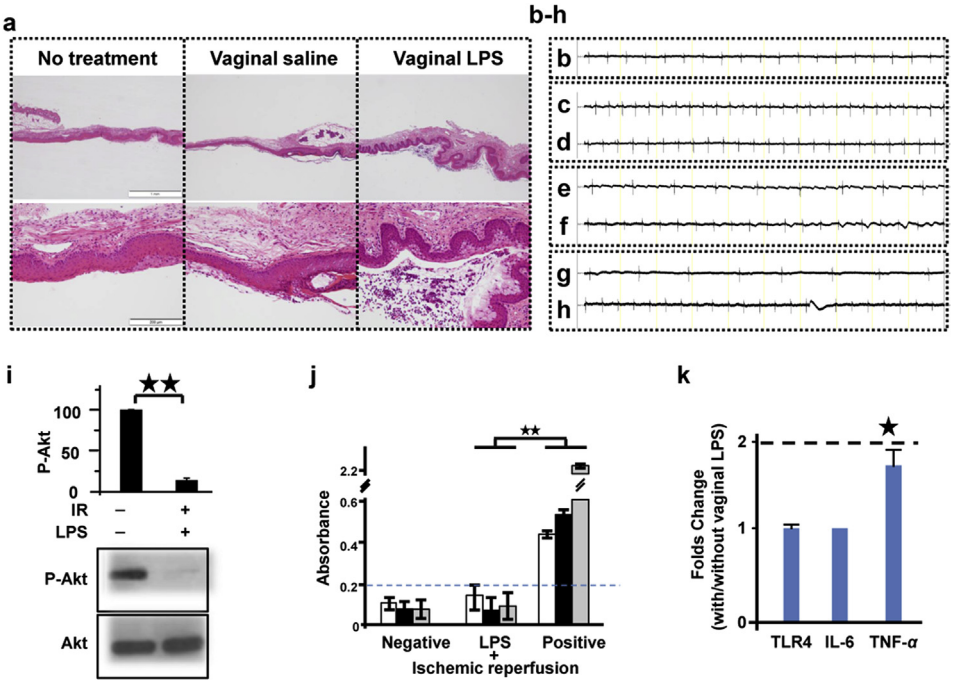

Fig. 2. Vaginal LPS induced local inflammation but not changes fetal heart rate in response to ischemic reperfusion. (a) Vaginal LPS induced inflammatory cell was present at the junction between the vagina and uterus. HE staining (20 x). Bar length: 100 μm. (b–h) Fetal electrocardiograph data of (b) mother mouse's heart beat rate, (c) 1st ischemia, (d) 1st reperfusion, (e) 2nd ischemia, (f) 2nd reperfusion, (g) 3rd ischemia and (h) 3rd reperfusion. (i) Phosphorylation of AKT was measured using total AKT under indicated conditions. ($n = 3$ fetuses derived from at least two pregnant mice). (j) IL-6 (white column), G-CSF (black column) and GM-CSF (grey column) were detected in the plasma of maternal mice within 30 min after IR. Compared with the positive controls and negative control, absorbance at 450 nm less than 0.20 was considered as negative ($n = 6$). LPS means the maternal plasma from 3 μg LPS group mice. (k) Vaginal LPS increased TNF-α mRNA level in fetal brain ($n = 5$ from at least 3 individual pregnant mice). This result suggested that vaginal LPS precondition sensitized the fetal brain via inflammatory reaction. Student's t test (two tailed for independent samples) was used for two group comparison, and Kruskal–Wallis one-way ANOVA was used for multiple group comparison. $p < 0.05$ indicated as a single star and $p < 0.01$ indicated as two stars.

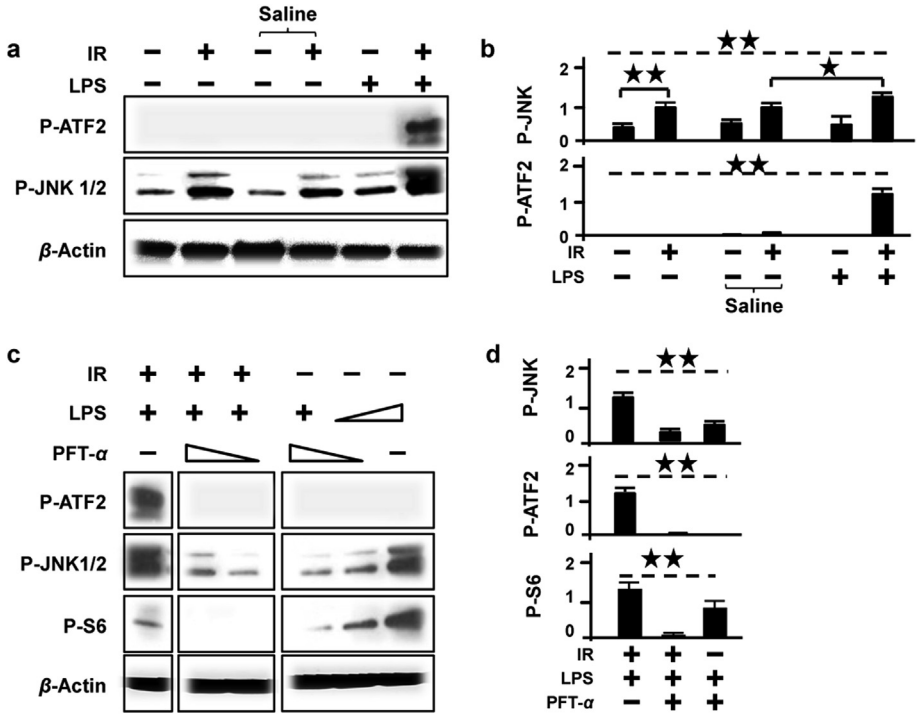

Fig. 3. Vaginal LPS induced Phosphorylation of ATF2 at Thr71 in response to ischemic reperfusion which was blocked by PFT-α in the fetal brain. (a) Only with vaginal LPS precondition, the phosphorylation of ATF2 at Thr71 appeared within 30 min response to ischemic reperfusion. (b) The summary of phosphorylation of JNK1/2 and ATF2 compared with beta-actin. ($n = 5$ fetuses from at least 3 individual pregnant mice). (c) Pre-treatment mice with PFT-α at 6 h (lane 3 and 5) or 3 h (lane 2 and 4) before ischemic reperfusion, it blocked phosphorylation of ATF2, completely. (d) The summary of Western blot data ($n = 5$ fetuses from at least 3 individual pregnant mice). Student's t test (two tailed for independent samples) was used for two group comparison, and Kruskal–Wallis one-way ANOVA was used for multiple group comparison. $p < 0.05$ indicated as a single star and $p < 0.01$ indicated as two stars.

injury [4,5,34]. Recently, evidence from animal model suggested that LPS induced inflammation has a neuroprotective role in the fetal brain [19,34]. Under vaginal LPS precondition, functional p53 in response to ischemic reperfusion who induced fetal brain damage triggered an ATF2-dependent rescue system [9,28,29] (Fig. 3a), because phosphorylated ATF2 become stable and recovers the balance between HIF-1α and P53 [32,33]. However, this repair process increased the vulnerability of hemorrhage (Fig. 1e).

Recently, there was a vast improvement in perinatal care, such as genetic investigation to diagnose congenital anomaly. However, the vulnerability of cerebral palsy remains to be about 2% in the world [20,35,36]. Severe cases of cerebral palsy were mainly occurred by cerebral hemorrhage during gestation. However, fetal brain hemorrhage is difficult to diagnose in uterus [19]. Until now, there is no real-time monitoring technology to identify these vulnerable patients. Moreover, little is known of the pathophysiology of cerebral hemorrhage during gestation making it harder to establish a prophylactic treatment [30]. We have attempted to propose the usage of FECG to monitor the fetal condition together with the evaluation of ATF2 as a biomarker. This combination will possibly allow us to understand the underlying mechanisms behind fetal brain damage.

5. Conclusion

Vaginal LPS administration increased the risk of brain hemorrhage in response to IR and triggered P53-dependent ATF2 phosphorylation.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.10.125.

Appendix A. Supplementary data

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