

Change in prostaglandin E synthases (PGESs) in microsomal PGES-1 knockout mice in a preterm delivery model

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Abstract

Most preterm deliveries are associated with infection and inflammation. Prostaglandin E₂ (PGE₂) is one of the most important mediators in the processes of inflammation, and is converted from PGH₂ by various kinds of PGE synthases (PGESs). Among PGESs, microsomal PGES-1 (mPGES-1) is known to be the most important subtype in the processes of inflammation. To evaluate the role of PGESs in preterm delivery, we used mPGES-1 knockout mice in a lipopolysaccharide (LPS)-induced preterm labor model. Unexpectedly, the duration of labor after LPS treatment was not statistically different between C57BL/6 wild-type mice and mPGES-1 knockout mice.

In wild-type mice, mPGES-1 mRNA and protein expression increased in the myometrium and fetal membrane after LPS treatment. In contrast, the expression of mPGES-2 or cytosolic PGES was not changed by LPS treatment. On mPGES-1 knockout mice, mPGES-2 increased by LPS treatment in myometrium. The present data indicate that mPGES-1 may be involved in LPS-induced preterm labor, but inhibition of mPGES-1 alone may not prevent preterm delivery, because mPGES-2 might compensate for the role of mPGES-1.

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Introduction

Preterm delivery is the leading cause of perinatal mortality and morbidity. Most preterm deliveries are associated with intrauterine infection. Prostaglandin E₂ (PGE₂) is thought to be a very important autacoid which regulates fever, swelling, pain, tumorigenesis, gastrointestinal protection, osteogenesis and wound healing (Funk 2001, Harris *et al.* 2002, Murakami & Kudo 2004). PGE₂ production from arachidonic acid, which is released by phospholipase A₂ from membrane phospholipids, is controlled by two rate-limiting steps (Funk 2001, Harris *et al.* 2002, Murakami & Kudo 2004). The first step is catalyzed by cyclooxygenase (COX), which converts arachidonic acid to the intermediate prostanoid PGH₂. Two isoforms of the COX enzymes have been identified: COX-1, which is a constitutive enzyme; and COX-2, which is induced by various stimuli, including cytokines, growth factors, tumor promoters and lipopolysaccharide (LPS). The second step is the terminal conversion reaction of PGH₂ to PGE₂, which is catalyzed by PGE synthase (PGES) (Jakobsson *et al.* 1999, Murakami *et al.* 2000, 2003). Nowadays, three isoforms of PGE synthase have been identified:

microsomal PGE synthase-1 (mPGES-1) (Jakobsson *et al.* 1999); microsomal PGE synthase-2 (mPGES-2) (Watanabe *et al.* 1999, Tanikawa *et al.* 2002); and cytosolic PGE synthase (cPGES) (Tanioka *et al.* 2000).

Among these three enzymes, mPGES-1, which was originally designated as membrane-bound glutathione S-transferase-1-like 1 (MGST1-L1), is the most important isozyme in infectious response (Jakobsson *et al.* 1999). This isozyme is thought to be coordinately induced with COX-2 on the perinuclear membrane and is functionally coupled with COX-2 in marked preference to COX-1 (Murakami *et al.* 2000). mPGES-1 expression is induced by pro-inflammatory stimuli in various tissues and cells, and is down-regulated by dexamethasone, accompanied by change in COX-2 expression and PGE₂ production (Murakami *et al.* 2000). COX-2 and mPGES-1, therefore, are thought to be essential components for PGE₂ biosynthesis under pathological conditions. mPGES-2 was originally extracted from bovine heart (Watanabe *et al.* 1999, Tanikawa *et al.* 2002). This isozyme was synthesized as a Golgi membrane-associated protein and by spontaneous cleavage of the N-terminal hydrophobic domain, and led to the formation of a truncated protein that was

Table 1 Primers used in the experiments

	Forward primer	Reverse primer	Genbank accession number	Base pair
COX-1	5'-gcatgtggctgtggatgca-3'	5'-ggcttgggttgaggcaga-3'	BC005573.1	388
COX-2	5'-acactctatcactggcacc-3'	5'-gaagggacacccctcacat-3'	NM011198.1	585
mPGES-1	5'-gagcccaccgcaacgacatg-3'	5'-cagatgggtggccacctccc-3'	BC024960	248
mPGES-2	5'-ccaggacggaggagatgaagtg-3'	5'-ccaaggctggatgtgagtg-3'	BC004846	307
cPGES	5'-atgcagcctgcttctgca-3'	5'-ttactccagatctggcat-3'	L24804	483
β -actin	5'-attgatggactccggtgacgg-3'	5'-atcttgatctcatggctag-3'	M12481.1	536

distributed in the cytosol with a trend to be enriched in the perinuclear region. mPGES-2 promoted PGE₂ production via both COX-1 and COX-2 in the immediate and delayed responses with modest preferences (Murakami *et al.* 2003). cPGES, which was identified as the heat shock protein 90 (HSP90)-associated protein p23, is thought to be constitutive and to be linked with COX-1 (Tanioka *et al.* 2000).

In previous studies, it has been reported that female mPGES-1 knockout mice showed no functional defects in reproductive behavior (Uematsu *et al.* 2002, Trebino *et al.* 2003). Here, to understand the role of mPGES-1 in preterm delivery due to the infectious response, we investigated whether or not the duration of labor in the LPS-induced preterm delivery model was affected, and also investigated the changes in expression patterns of various PGESs in preterm delivery.

Materials and Methods

Animals

C57BL6 mice (wild-type) were obtained from the Sankyo laboratory. The mPGES-1 knockout mice (C57BL6 \times 129/SvJ background) were as described previously (Uematsu *et al.* 2002, Kamei *et al.* 2004). As previously demonstrated, normal mPGES-1 protein was not produced in these knockout animals (Uematsu *et al.* 2002). Female mice (10–12 weeks old) were mated overnight to obtain pregnant females. Mating was confirmed by the presence of seminal plug or sperm. Seminal plug day was designated as day 0 and represented the beginning of the gestational period. The duration of pregnancy in both wild-type and mPGES-1 knockout mice was 19 days. There were no differences between each group statistically. All experiments were performed with the approval of the Tokyo Medical and Dental University.

Agents

LPS and *Escherichia coli* 055:B were purchased from Sigma. Rabbit anti-mPGES-1 polyclonal antibodies and rabbit anti-cPGES antibodies were purchased from Cayman

Chemicals (Ann Arbor, MI, USA). Rabbit anti-mPGES-2 antibodies were produced in our laboratory. The antibody against mPGES-2 was not cross-reacted with mPGES-1 and cPGES. Mouse anti- α tubulin polyclonal antibodies were purchased from Sigma. Anti-rabbit IgG antibodies and anti-mouse IgG antibodies, and enzyme immunoassay kits for PGE₂ were purchased from Amersham.

Preterm delivery model with LPS treatment

LPS or saline were injected i.p. at 5 mg/kg (150–170 μ g per mouse) in the mice on day 15. The duration of labor after LPS administration was counted every 0.5 h.

RNA extraction

Total RNA was extracted from tissue specimens after homogenization in Trizol reagent (Invitrogen) according to the manufacturer's instruction. The quantity and quality of the RNA was verified by spectrophotometry.

Reverse transcription (RT)-PCR

One microgram of total RNA was used as a template for cDNA synthesis. The cDNA was prepared using random primers and River Tra Ace (TOYOBO, Osaka, Japan). PCR primers were designed and synthesized on the basis of reported mouse cDNA sequences for COX-1, COX-2, mPGES-1, mPGES-2, cPGES and β -actin (Table 1). Each PCR reaction was performed in a 25 μ l mixture containing Ex Taq, dNTP (TAKARA, Tokyo, Japan) and primers. Cycling conditions were as follows: denaturation for 40 s at 94 °C, annealing for 30 s at 62 °C and extension for 45 s at 72 °C for COX-1 (28 cycles), COX-2 (32 cycles) and β -actin (22 cycles); denaturation for 40 s at 94 °C, annealing for 30 s at 58 °C, extension for 45 s at 72 °C for mPGES-1 (30 cycles) and mPGES-2 (30 cycles); and denaturation for 40 s at 94 °C, annealing for 30 s at 50 °C, extension for 45 s at 72 °C for cPGES (25 cycles).

After amplification, the PCR products were resolved by 1.2% agarose gel electrophoresis and visualized by ethidium bromide (Sigma).

Table 2 Primers and probe pairs used in the experiments

	Forward primer	Reverse primer	Probe 1	Probe 2
mPGES-1	5'-accgcaacgacatggag-3'	5'-ggttcagctgccagga-3'	5'-cgaagccgaggaagaggaaagatagatt-3'	5'-agggttggtcccaggaatgagtac-3'
mPGES-2	5'-gcgacactcacatccag-3'	5'-gtggaccagagacaaatgact-3'	5'-gatggagcgggccattgaagaagc-3'	5'-ccctcggtgatcatgtcaaccc-3'
β -actin	5'-cctgtatcctctgctgta-3'	5'-ccatctcctgctgaagtct-3'	5'-ctgagagggaaatcgtgctgacatc-3'	5'-cgtggctacagcttcaccaccaca-3'

Real-time quantitative PCR

Quantification of relative mRNA abundance for mPGES-1 and mPGES-2 was performed by real-time PCR amplification in a Light Cycler (Roche) instrument using hybridization probe methods. Each hybridization probe was designed and synthesized on the basis of mouse sequences reported by Japanese gene research laboratories. The primers and probe pairs are shown in Table 2. A 20 μ l PCR reaction was used and included quantitative PCR master mix (Qiagen), primers, probes and reversed transcribed cDNA. For quantification, standard curves were described using serial dilutions of the appropriate plasmid. Each PCR product was confirmed by size determination using agarose gel electrophoresis. Data obtained from the Light Cycler were normalized against β -actin.

Protein preparation

Tissues were harvested and frozen in liquid nitrogen and kept at -80°C until processed. Tissues were homogenized in 0.25% sucrose, 5 mM EDTA, 3% Triton \times 100 and protease inhibitor cocktail (Sigma). The homogenates were centrifuged at 10 000 g for 10 min. A portion of supernatants was used to prepare the microsomal fraction by centrifugation at 56 000 g for 60 min. The supernatants were used for cytosolic fractions. The pellets were solubilized in 250 mM sucrose, 5 mM EDTA, 3% Triton \times 100 and protease inhibitor cocktail. Protein content was measured using a protein assay kit (BioRad) using bovine albumin as standard. Fractions were kept at -20°C until further analysis.

SDS-PAGE and immunoblotting

Twenty microgram of microsomal protein extracts were subjected to electrophoresis on 15% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked in TBS and 0.1% Tween20 containing 5% dry milk (TBS-T) and then incubated with mPGES-1 (\times 1000), mPGES-2 (\times 5000), cPGES (\times 1000) and α -tubulin (\times 5000).

After incubation with primary antibody, the membrane was washed in TBS-T four times for 15 min each and then exposed to horseradish peroxidase-conjugated anti-mouse

or anti-rabbit IgG secondary antibody (\times 5000) at 4°C for 2 h. Detections were performed using ECL plus western blotting detection reagents (Amersham). Signal strength was digitized and quantified using RAS1000 plus (Fuji Film, Tokyo, Japan).

Statistics

Data are expressed as means \pm s.d. and were compared using the Mann-Whitney U -test. A P value of less than 0.05 was considered to be statistically significant.

Results

Duration of labor after LPS treatment in mPGES-1 knockout mice

The duration of labor in LPS-treated wild-type mice was 10.95 ± 0.59 h (mean \pm s.d.), and that in LPS-treated mPGES-1 knockout mice was 11.60 ± 0.93 h (mean \pm s.d.). There was no significant difference between mPGES-1 knockout mice and wild-type mice (Fig. 1).

Gene expression of mPGES-1, mPGES-2 and cPGES in LPS-treated pregnant mice

It was observed by RT-PCR that in the myometrium, placenta and fetal membrane in the wild-type mice, COX-2 mRNA production increased at 6 h after LPS treatment, but COX-1 and cPGES-1 mRNA were not influenced by LPS treatment. mPGES-1 mRNA expression was elevated in the myometrium and placenta, while mPGES-2 mRNA expression in these tissues was not affected by LPS treatment (Fig. 2a).

To confirm the gene expression of mPGES-1 and mPGES-2 in each tissue, we conducted quantitative real-time PCR. mPGES-1 mRNA expression in the myometrium and fetal membrane increased at 6 h after LPS treatment, but that in placenta tended to increase (Fig. 3a). mPGES-2 expression in each tissue, however, did not change in these tissues following LPS treatment (Fig. 3b).

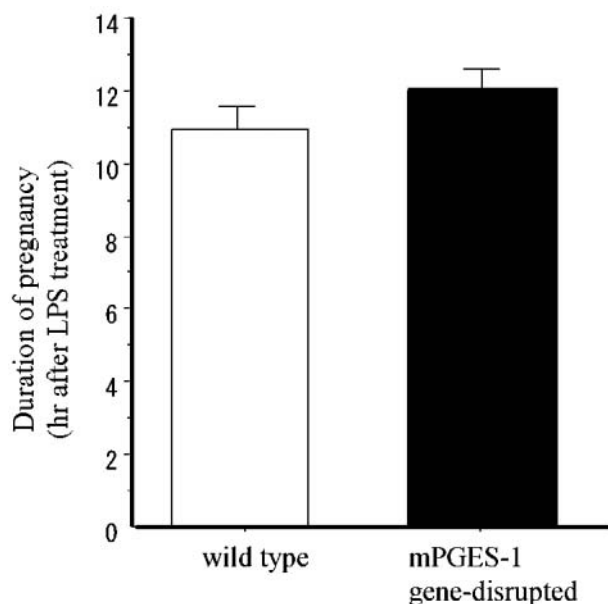


Figure 1 Duration of labor after LPS treatment in wild-type mice and mPGES-1 knockout mice. LPS or saline were injected i.p. at 5 mg/kg (150–170 µg per mouse) in mice. The duration of labor after LPS administration was counted every 0.5 h. Values are presented as means \pm s.d. Wild-type mice, $n=11$; mPGES-1 knockout mice, $n=7$.

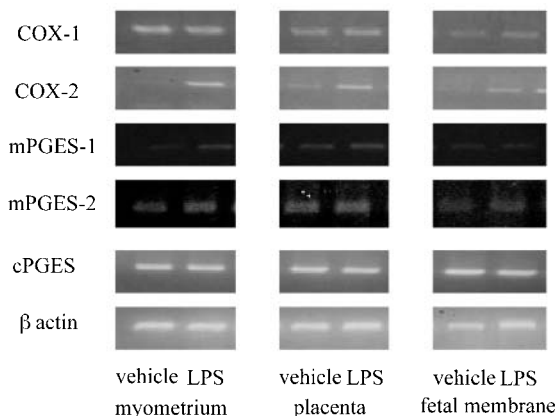
Expression of mPGES-1 and mPGES-2 proteins after LPS treatment

mPGES-1 and mPGES-2 protein levels in the myometrium, placenta and fetal membrane were detected by western blotting, and were normalized by α -tubulin. In wild-type mice, in the myometrium mPGES-1 protein level as well as mRNA expression increased at 6 h after LPS treatment (Fig. 4a); levels in the placenta tended to increase, but not this was not significant. In contrast, mPGES-2 protein did not increase after LPS treatment in the myometrium, placenta and fetal membrane of wild-type mice (Fig. 4b).

Gene and protein expression of mPGES-2 after LPS treatment in mPGES-1 knockout mice

RT-PCR showed that mRNA expression of COX-2 and mPGES-2 in the myometrium of mPGES-1 knockout mice increased at 6 h after LPS treatment, but COX-1 and cPGES-1 mRNA were not influenced by LPS treatment (Fig. 2b). The upregulation of mPGES-2 mRNA in the myometrium, but not in placenta and fetal membrane, at 6 h after LPS treatment was confirmed by quantitative real-time PCR (Fig. 5). The difference in expression of mPGES-2 mRNA and proteins in the myometrium between wild-type and mPGES-1 knockout mice was more clearly observed at labor after LPS treatment (Fig. 6).

a) Wild-Type



b) mPGES-1 gene-disrupted

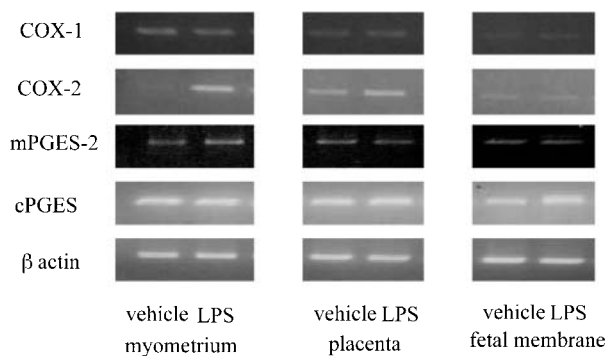


Figure 2 Gene expression of COX-1, COX-2, mPGES-1 and mPGES-2 in the myometrium, placenta and fetal membrane at 6 h after LPS treatment in (a) wild-type mice and (b) mPGES-1 knockout mice. At 6 h after LPS treatment, the myometrium, placenta and fetal membrane were isolated from (a) wild-type mice and (b) mPGES-1 knockout mice, and 1 µg total RNA in each tissue was used for RT-PCR.

Discussion

In the majority of cases, preterm birth is associated with microbial invasion of the chorion, amnion, endometrium or placenta, with inflammatory cytokines in the amniotic fluid, or with preterm fetal membranes. Currently, non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been shown to be effective in delaying labor in the short-term (48–72 h), suggesting that PGs are involved in preterm birth. Among several PGs, PGE₂ is the most important mediator that plays a variety of roles in biological events (Funk 2001, Harris *et al.* 2002, Murakami *et al.* 2004). Moreover, Cook *et al.* (2000) reported that an increase in PGF_{2 α} was not necessary for preterm labor and that increases in COX-2 at the time of preterm birth might underlie PGE₂ change. Among PGE

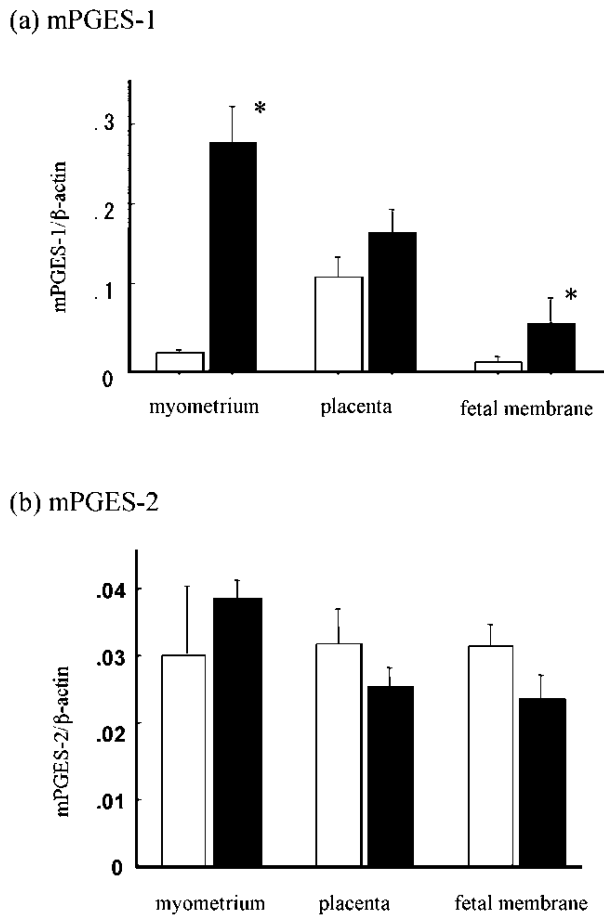


Figure 3 Quantitative analysis of gene expression of (a) mPGES-1 and (b) mPGES-2 in the myometrium, placenta and fetal membrane at 6 h after LPS treatment in wild-type mice. At 6 h after LPS treatment, the myometrium, placenta and fetal membrane were isolated from the wild-type mice. Open bars indicate control and filled bars show LPS-treatment. cDNA extracted from the tissues was measured by Light Cycler real-time PCR to assess the level of mPGES-1 and mPGES-2 mRNA. Data obtained from the Light Cycler were normalized against β -actin. Values are presented as means \pm s.d. ($n=5$). * $P<0.01$.

synthases, mPGES-1 is induced by pro-inflammatory stimuli in various tissues and cells, and is down-regulated by dexamethasone, indicating that COX-2 and mPGES-1 are essential for PGE₂ biosynthesis under pathological conditions. These reports led us to the hypothesis that mPGES-1 is involved in preterm labor. Therefore, we investigated whether the duration of labor after LPS treatment was elongated in mPGES-1 knockout mice. However, unexpectedly there was no significant difference in the duration of labor between mPGES-1 knockout mice and wild-type mice. In previous investigations, the normal reproductive systems in mPGES-1 knockout mice also showed no abnormality (Uematsu *et al.* 2002, Trebino *et al.* 2003). In contrast, COX-2 knockout mice

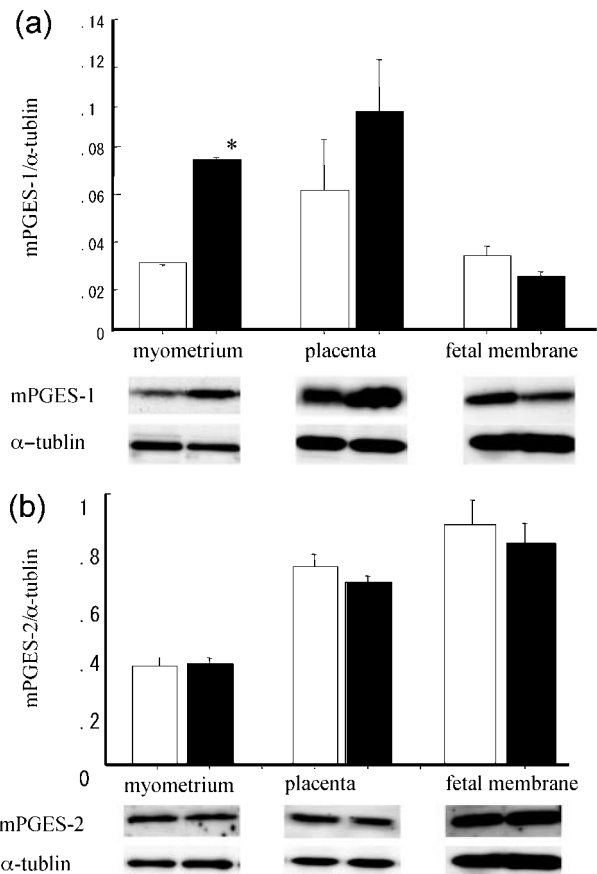


Figure 4 Representative western blot of (a) mPGES-1 and (b) mPGES-2 protein in wild-type mice and quantification by densitometry. Proteins in the microsomal fraction isolated from the myometrium, placenta and fetal membrane in wild-type mice at 6 h after LPS treatment were subjected to western blotting and the data obtained from densitometry were normalized against α -tubulin. Open bars indicate control and filled bars show LPS-treatment. The densitometric results in four independent experiments represent the means \pm s.d. * $P<0.05$.

showed female reproductive abnormalities with defects in ovulation, fertilization, implantation and decidualization (Lim *et al.* 1997), and PGE₂ receptor, EP2 knockout mice showed a defect in ovulation (Kennedy *et al.* 1999, Tilley *et al.* 1999).

To examine the contribution of other PGESs in LPS-induced preterm delivery, the changes in PGES expression pattern were observed in wild-type mice. However, only the expression of mPGES-1 increased in the myometrium and fetal membrane after LPS treatment, and the expression of mPGES-2 and cPGES were not changed after LPS treatment. In contrast to the wild-type mice, elevated mPGES-2 mRNA in the myometrium after LPS treatment was observed in mPGES-1 knockout mice. This indicates that mPGES-2 may compensate functionally for the loss of mPGES-1. This compensation has been

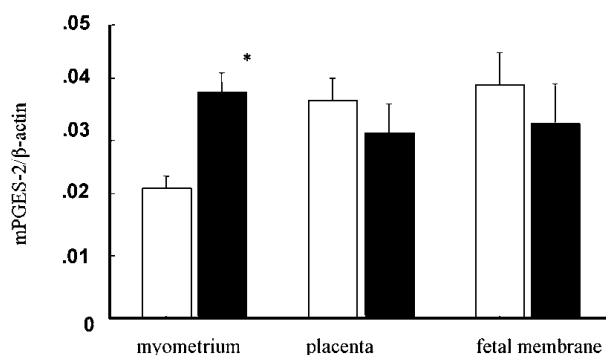


Figure 5 Quantitative analysis of gene expression of mPGES-2 in the myometrium, placenta and fetal membrane at 6 h after LPS treatment in mPGES-1 knockout mice. At 6 h after LPS treatment, the myometrium, placenta and fetal membrane were isolated from mPGES-1 knockout mice. Open bars indicate control and filled bars indicate LPS-treatment. cDNA extracted from the tissues was measured by Light Cycler real-time PCR to assess the level of mPGES-2 mRNA. Open bars indicate control and filled bars indicate LPS-treatment. Data obtained from the Light Cycler were normalized against β -actin. Values are presented as means \pm s.d. ($n=6$). * $P<0.05$.

reported in COX isozymes: interleukin-1 β could induce PGE₂ synthesis in lung fibroblasts derived from COX-1 knockout mice as well as COX-2 knockout mice (Kirtikara *et al.* 1998). It has been reported that mPGES-2 expression was not increased appreciably during tissue inflammation or damage (Murakami *et al.* 2003). As shown in this paper, however, mPGES-2 expression increased following LPS treatment under conditions where mPGES-1 was reduced. The present data demonstrated that mPGES-1 maybe involved in LPS-induced preterm labor, but inhibition of mPGES-1 alone may not prevent preterm delivery, because mPGES-2 might compensate for the role of mPGES-1.

In a recent study, a COX-2-specific inhibitor, as well as indomethacin, induced premature constriction of the fetal ductus arteriosus (Loftin *et al.* 2002). Therefore, the inhibition of mPGES-1 may be a benefit for preterm delivery, but up to now the specific inhibitors of mPGES-1 have not been developed. In this paper, therefore, we used mPGES-1 knockout mice and investigated whether the duration of labor after LPS treatment was elongated. However, mPGES-2 compensated functionally for the loss of mPGES-1, and we failed to show the beneficial effects of mPGES-1 inhibition for preterm labor. Thus, further investigation using mPGES-1 and mPGES-2 double knockout mice is warranted to disclose the role of PGESs in preterm delivery.

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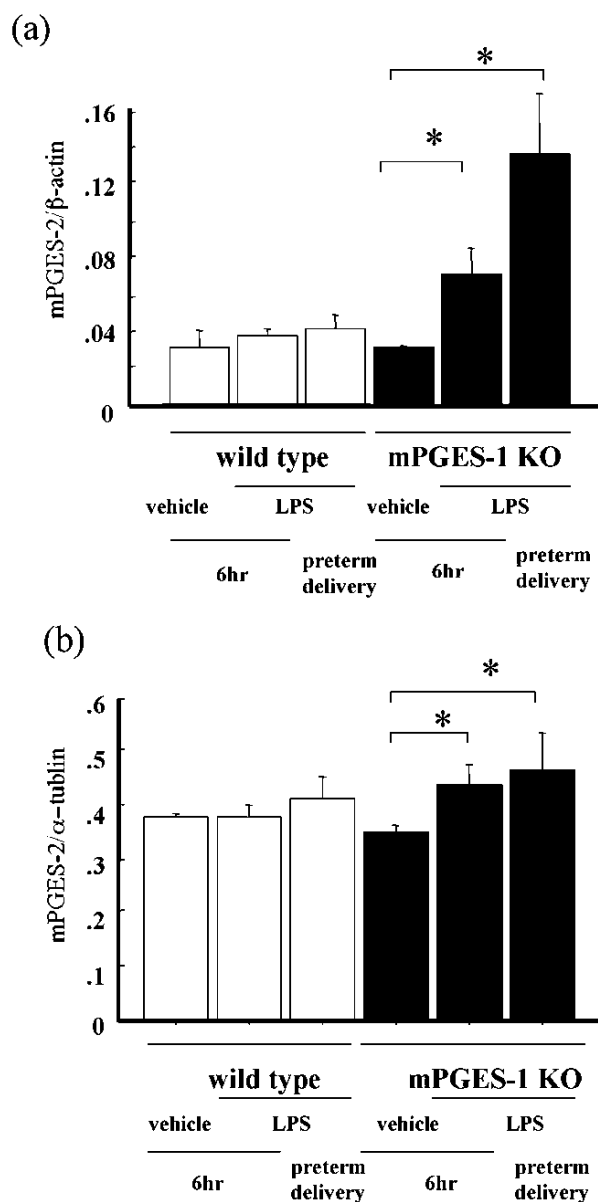


Figure 6 Comparison of (a) mRNA and (b) protein of mPGES-2 expression in the myometrium after LPS treatment between the wild-type mice and mPGES-1 knockout (KO) mice. At 6 h after LPS treatment and at the time of preterm delivery after LPS treatment, the myometrium was isolated from wild-type mice and mPGES-1 knockout mice. (a) cDNA extracted from the tissues was measured by Light Cycler real-time PCR to assess the level of mPGES-2 mRNA ($n=5$). (b) Proteins in the microsomal fraction were applied to western blotting and the data obtained from densitometry were normalized against α -tubulin. The densitometric results of four independent experiments are given as means \pm s.d. * $P<0.05$.

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The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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