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 PG 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 C57BL6 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.


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 prostaglandin 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) (Jakobbsson et al. 1999, Murakami et al. 2000, 2003). Nowadays, three isoforms of PGE synthase have been identified: microsomal PGE synthase-1 (mPGES-1) (Jakobbsson 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-transferase1-like 1 (MGST1-L1), is the most important isozyme in infectious response (Jakobbsson 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...
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. 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.

**Materials and Methods**

**Animals**

C57BL6 mice (wild-type) were obtained from the Sankyo laboratory. The mPGES-1 knockout mice (C57BL6 × 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 µg 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 µl 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 (22 cycles), COX-2 (24 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 1

<table>
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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<th>Base pair</th>
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<td>COX-2</td>
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<td>cPGES</td>
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<td>β-actin</td>
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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 shown in Table 2. A 20 µl PCR reaction was used and included quantitative PCR master mix (Qiagen), primers, probes and reverse 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 was 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 × 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 × 100 and protease inhibitor cocktail. Protein context 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 (× 1000), mPGES-2 (× 5000), cPGES (× 1000) and α-tublin (× 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 (× 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 ± 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 ± S.D.), and that in LPS-treated mPGES-1 knockout mice was 11·60 ± 0·93 h (mean ± 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 mouse

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).
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).

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, PGE$_2$ 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 PGE$_{2\alpha}$ was not necessary for preterm labor and that increases in COX-2 at the time of preterm birth might underlie PGE$_2$ change. Among PGE

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 ± s.d. Wild-type mice, n=11; mPGES-1 knockout mice, n=7.

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.
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 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 shown by previous studies.

**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. Open bars indicate control and filled bars show LPS-treatment. Data obtained from the Light Cycler were normalized against β-actin. Values are presented as means ± s.d (n=5). *P<0.01.

**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. The densitometric results in four independent experiments represent the means ± s.d *P<0.05.
reported in COX isozymes: interleukin-1β 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.

**Acknowledgement**

The authors thank Dr Shizuo Akira, Osaka University, for providing the mPGES-1 knockout mice.

**Funding**

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


Received 29 August 2005
Accepted 1 September 2005
Made available online as an Accepted Preprint 13 September 2005