Coupling of COX-1 to mPGES1 for prostaglandin E\textsubscript{2} biosynthesis in the murine mammary gland

Subhashini Chandrasekharan,* Nicholas A. Foley,* Leigh Jancia,* Patsy Clark,† Laurent P. Audoly,† and Beverly H. Koller\textsuperscript{1,*}

Department of Genetics,* University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; and Merck Frost Centre for Therapeutic Research,† Kirkland, Quebec H9H 3L1, Canada

Abstract The mammary gland, like most tissues, produces measurable amounts of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), a metabolite of arachidonic acid produced by sequential actions of two cyclooxygenases (COX-1 and COX-2) and three terminal PGE synthases: microsomal prostaglandin E\textsubscript{2} synthase-1 (mPGES1), mPGES2, and cytosolic prostaglandin E\textsubscript{2} synthase (cPGES). High PGE\textsubscript{2} levels and COX-2 overexpression are frequently detected in mammary tumors and cell lines. However, less is known about PGE\textsubscript{2} metabolic enzymes in the context of normal mammary development. Additionally, the primary COX partnerships of terminal PGE synthases and their contribution to normal mammary PGE\textsubscript{2} biosynthesis are poorly understood. We demonstrate that expression of COX-1, generally considered constitutive, increases dramatically with lactogenic differentiation of the murine mammary gland. Concordantly, total PGE\textsubscript{2} levels increase throughout mammary development, with highest levels measured in lactation and breast milk. In contrast, COX-2 expression is extremely low, with only a modest increase detected during mammary involution. Expression of the G\textsubscript{1}-coupled PGE\textsubscript{2} receptors, EP2 and EP4, is also temporally regulated, with highest levels detected at stages of maximal proliferation. PGE\textsubscript{2} production is dependent on COX-1, as PGE\textsubscript{2} levels are nearly undetectable in COX-1-deficient mammary glands. Interestingly, PGE\textsubscript{2} levels are similarly reduced in lactating glands of mPGES1-deficient mice, indicating that PGE\textsubscript{2} biosynthesis results from the coordinated activity of COX-1 and mPGES1. We thus provide evidence for the first time of functional coupling between COX-1 and mPGES1 in the murine mammary gland in vivo.—Chandrasekharan, S., N. A. Foley, L. Jancia, P. Clark, L. P. Audoly, and B. H. Koller. Coupling of COX-1 to mPGES1 for prostaglandin E\textsubscript{2} biosynthesis in the murine mammary gland. J. Lipid Res. 2005, 46: 2636–2648.

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Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), a lipid mediator produced by most mammalian tissues, regulates multiple biological processes under both normal and pathological conditions. In addition to being a key mediator of inflammation, PGE\textsubscript{2} was recently demonstrated to play an important role in epithelial cell physiology, particularly in gastrointestinal tissues. The biosynthesis of PGE\textsubscript{2} is achieved by sequential actions of three groups of enzymes. First, membrane-bound and secretory phospholipase A\textsubscript{2} isoenforms convert phospholipids to arachidonic acid (AA). Next, the cyclooxygenases (COXs) convert AA into the unstable intermediate, prostaglandin endoperoxide (PGH\textsubscript{2}). Finally, terminal PGE\textsubscript{2} synthase (PGES) enzymes isomerize PGH\textsubscript{2} into PGE\textsubscript{2}. Two COX enzymes, COX-1 and COX-2, catalyze the rate-limiting step in PGE\textsubscript{2} biosynthesis (i.e., conversion of AA to PGH\textsubscript{2}). COX-1 and COX-2, in addition to having different subcellular localizations, also have different tissue expression profiles. COX-1 is primarily associated with constitutive or “housekeeping” functions in normal tissues. In contrast, with the exception of some organs such as the kidney, testis, and the central nervous system, COX-2 expression is extremely low in most normal tissues and is induced by growth factors, cytokines, and proinflammatory stimuli. High COX-2 expression is also associated with pathological conditions, such as tissue damage and malignant transformation of gastrointestinal and mammary epithelium. The distinct functional roles of COX-1 and COX-2 in vivo are further supported by different physiological defects observed in COX-1-deficient (COX-1\textsuperscript{−/−}) and COX-2-deficient (COX-2\textsuperscript{−/−}) mice (1, 2).

To date, three different genes with PGES activity have been cloned (3). The first PGES, microsomal prostaglan-
din E2 synthase-1 (mPGES1), was isolated as a microsomal protein and is a member of the MAPEG (for membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily (4, 5). mPGES1 expression is low in most normal tissues, although abundant and constitutive expression is detected in a limited number of organs, such as the lung, kidney, and reproductive organs. Interestingly, mPGES1 expression is increased in a number of cancers, including lung, gastric, and colorectal tumors, suggesting that increases in PGE2 levels during ovulation may be primarily regulated by mPGES1 (17). However, little is known about the contribution of mPGES1 or its COX partnerships for PGE2 biosynthesis during normal mammary homeostasis in vivo. Mammary gland development is characterized by the coordination of multiple biological processes that results in regulated phases of cellular proliferation, differentiation, and apoptosis. We have examined the expression patterns of all terminal PGE synthases and COXs during different stages of murine mammary gland development. We have also determined the expression levels of all PGE2 receptor isoforms, EP1–EP4, at these stages. In this study, we report that high PGE2 levels detected during lactation result from a dramatic induction of COX-1 RNA and protein. We also demonstrate that the Gs-coupled receptors EP2 and EP4 have similar expression profiles, with maximal levels detected during the proliferative phase of pregnancy. PGE2 biosynthesis in the mammary gland is dependent on COX-1, as COX-1−/− mice, although able to lactate efficiently, have no detectable PGE2 in mammary tissue or breast milk. PGE2 biosynthesis also appears to be primarily dependent on mPGES1, because PGE2 levels are reduced significantly in lactating glands of mPGES1−/− mice. In contrast, PGE2 levels measured in lactating mammary glands of mPGES2−/− mice were not significantly different from those of wild-type controls. These findings provide evidence that although all three terminal PGE synthases are expressed in the murine mammary gland, during normal mammary development COX-1 and mPGES1 are the predominant functional partners in vivo.

**MATERIALS AND METHODS**

**Animals**

C57BL/6 and B6/D2 females were purchased from Jackson Laboratories and Taconic Laboratories, respectively. Eight to 10 week virgin females were mated for isolation of mammary glands at gestation, lactation, and involution. Pregnancy was confirmed by detection of vaginal plugs. The first day of plug visualization was counted as day 0.5. COX-1−/− females were generated by intercrosses of COX-1+/− mice on the 129/B6D2 mixed genetic background. C57BL/6 EP2−/− congenic pairs were generated by backcrossing EP2−/− mice to the C57BL/6 strain for 12 generations (18). mPGES1−/− mice were maintained on the DBA/1lacJ background. mPGES2−/− and mPGES2+/− mice were generated by heterozygous intercross and were on the 129/B6D2 mixed genetic background. All animal colonies were maintained in accordance with institutional animal guidelines.

**Measurement of PGE2**

The inguinal mammary glands without the lymph nodes were quickly dissected from each animal at the chosen developmental stage and snap-frozen in liquid nitrogen. Approximately 150 mg of frozen tissue was pulverized and then homogenized in ice-cold PBS containing 1 mM EDTA and 10 μM indomethacin. Homogenates were mixed with chilled 75% ethanol and treated with

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acetic acid to precipitate proteins. Prostaglandins were purified from tissue supernatants using Amprec Octadecl C18 mini columns (Amersham Biosciences) according to the manufacturer’s instructions. Prostaglandins were eluted with ethyl acetate, evaporated over N₂ at 40°C, and resuspended in ELISA assay buffer. Total (intracellular and secreted) steady-state PGE₂ levels were measured using the PGE₂ Monoclonal ELISA Kit (Neogen Corp.), which detects PGE₂ levels in the range of 0.10–4.00 ng/ml. ELISA was performed in duplicate for each sample. The cross-reactivity below. Isomeric analogs of PGE₂, such as 8-iso-PGE₂ and 11-iso-PGE₂, however, were not separated. The amounts of PGE₂ measured by ELISA could include, in some part, the levels of isomeric compounds detected by this antibody. For measurement of PGE₂ levels in breast milk, lactating females were mildly anesthetized and milk let down was stimulated by intraperitoneal administration of oxytocin. Aliquots (250 μl) of milk were collected and diluted in 1X PBS/EDTA containing 10 μM indomethacin. PGE₂ was extracted as described above. The lower limit of detection for PGE₂ was 0.2 ng/ml milk. Measurement of prostanoids, PGE₂, PGD₂, PGF₂, 6-keto-PGF₁α, and thromboxane B₂ (TXB₂) by liquid chromatography-mass spectrometry (LC-MS) was performed as described previously. (19) The lower limit of detection for PGE₂ was 0.0902 ng/mg protein. A representative chromatogram for the PGE₂ standard with retention times for PGE₂ (6.49) and PGD₂ (6.75) is shown in Fig. 5A below. Isomeric analogs of PGE₂, such as 8-iso-PGE₂ and 11-β-PGE₂, however, were not separated. The amounts of PGE₂ measured by LC-MS, therefore, potentially include the levels of these isomeric compounds as well (see Fig. 5D below).

**Isolation of total RNA and Northern blotting**

Inguinal mammary glands were dissected from three C57BL/6 females for each developmental stage: virgin, gestation, lactation, and involution. Samples were pooled, snap frozen in liquid nitrogen, divided randomly into three aliquots, and stored at −80°C. Pulverized frozen tissue was homogenized in RNA-Bee reagent (Tel-Test, Inc.) and total RNA was isolated according to the manufacturer’s instructions. Twenty micrograms of total RNA per sample was electrophoresed on 1.2% (w/v) formaldehyde-agarose gels. RNA was then transferred to nitrocellulose (Immobilon NC; Amersham Biosciences) or nylon (Hybond-XL; Amersham Biosciences) membranes by capillary transfer overnight. Northern blots were hybridized with [³²P]dCTP (Amersham Biosciences) membranes by capillary transfer overnight. Northern blots were hybridized with [³²P]dCTP (Amersham Biosciences) membranes by capillary transfer overnight. Membranes were blocked in 5% nonfat milk in 1X PBS containing 0.05% Tween 20 for 1 h at room temperature. Goat anti-mouse COX-1 antibody was used at a 1:200 dilution (Santa Cruz Biotechnology) and mouse anti-β-actin (Sigma) was used at a dilution of 1:5,000, and goat anti-mouse secondary antibody (Pierce, Ltd.) was used at a 1:10,000 dilution.

**Real-time quantitative RT-PCR analysis**

Total RNA was extracted from frozen tissue aliquots for each stage of mammary gland development as described above. Thirty to 50 μg of total RNA was further purified using Qiagen RNAeasy columns (Qiagen), and RNA integrity was evaluated using an Agilent 2100 Bioanalyzer apparatus. For each sample, cDNA was generated from 5 μg of purified total RNA using Multiscribe Reverse Transcriptase and random primers provided in the High-Capacity cDNA Archive Kit, according to the manufacturer’s instructions (Applied Biosystems). Five nanograms of cDNA was used in amplification reactions performed using the Taqman PCR Universal Master Mix kit (Applied Biosystems) according to the manufacturer’s instructions. All amplifications were performed on a Stratagene Mx300P cycler. Expression of mPGES1, mPGES2, dPGES, and COX-1 was detected using the following Taqman primer and probe sets (Applied Biosystems): COX-1, Mm00477214_m1; dPGES, Mm00727367_s1; mPGES1, Mm00452105_m1; mPGES2, Mm00460181_m1; PGDH, Mm00515121_m1. Expression levels were normalized to murine β₂-microglobulin used as the internal reference: Mm00437762 (Applied Biosystems). Expression of COX-2 at different stages of mammary gland development (Fig. 1C) was measured by real-time quantitative PCR and normalized to the mouse β-actin gene (internal reference) for each developmental stage. Primers and probes for β-actin have been described previously (20). The following primers were used for the amplification of COX-2: forward, 5’ GTG CCA ATT GCT GTA CAA GC 3’; reverse, 5’ ATG ATG TGT ACG GCT TCA GG 3’. All reverse transcription and amplification reactions for COX-2 were performed as described previously (20). Quantification of samples was performed using the comparative Ct (ΔΔCt) method for all of the above genes, as described in the Assays-on-Demand Users Manual (Applied Biosystems). Changes in expression levels during stages of pregnancy, lactation, and involution were calculated by the formula x = 2<sup>−ΔΔCt</sup> and expressed as fold changes compared to the resting virgin (10 week) mammary gland.

**Isolation of protein and Western blotting**

Total protein was isolated from inguinal mammary glands at different developmental stages by homogenizing frozen tissue aliquots in lysis buffer (50 mM HEPES, 0.15 M NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 10% glycerol containing freshly added protease inhibitors: 500 μM Na₂VO₄, 50 μM Na₃MoO₄; 10 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprogin, and 100 μg/ml PMSF). Homogenates were centrifuged at 14,000 rpm for 5 min at 4°C, and protein content of the supernatant was measured using BCA reagent (Pierce, Ltd.). Two hundred microliter aliquots of total protein were denatured by boiling at 95°C for 5 min and electrophoresed on 8% SDS-PAGE gels. Proteins were transferred using the Transblot™ apparatus (Bio-Rad) to Immobilon™ polyvinylidene difluoride membranes (Millipore) by electrolytic transfer. Membranes were blocked in 5% nonfat milk in 1X PBS containing 0.05% Tween 20 for 1 h at room temperature. Goat anti-mouse COX-1 antibody was used at a 1:200 dilution (Santa Cruz Biotechnology). Blots were incubated with primary antibodies diluted in 5% nonfat milk in 1X PBS containing 0.05% Tween 20 for 1–2 h at room temperature. Donkey anti-goat secondary antibodies (Santa Cruz Biotechnology) were diluted 1:10,000 in 1X PBS containing 0.05% Tween 20 and applied to membranes for 30 min at room temperature. Membranes were incubated with ECL reagent, and signal was detected by autoradiography after exposure to Hyperfilm™ (Amersham Biosciences). Mouse anti-β-actin (Sigma) was used at a dilution of 1:5,000, and goat anti-mouse secondary antibody (Pierce, Ltd.) was used at a 1:10,000 dilution.

**Densiometric analysis**

 Autoradiographs were scanned, and density of signal for bands of interest was measured using Image-Quant software. To generate normalized fold changes in gene expression during murine mammary gland development, the signal density of GAPDH was measured for each sample in the blot. Because the representation of housekeeping gene RNAs decreases compared to milk protein RNAs in the late-pregnant and lactating mammary gland, we determined the “mean” GAPDH expression level per blot. A correction factor based on this mean was then applied to each sample to get normalized “corrected” expression levels for every gene at each developmental stage. The expression levels at pregnancy, lactation, and involution stages were then expressed.
as fold changes compared with the levels detected in the resting virgin (10 week) mammary tissue.

**Statistical analysis**

All statistical analyses were performed applying the appropriate statistical tests mentioned in Results using the JMPIN (version 5.1) statistical software package (SAS).

**RESULTS**

**PGE₂ biosynthetic enzymes are differentially expressed during normal mammary gland development**

Total steady-state PGE₂ concentrations in the mammary gland were first measured at various developmental stages, including the mature virgin gland, early pregnancy, mid...
pregnancy, lactation, and involution, by ELISA. PGE₂ levels increased modestly during pregnancy compared to the virgin gland. However, levels increased dramatically during lactation (~19-fold higher at day 4 lactation compared with day 17 pregnancy) compared with even late gestational time points (Fig. 1A). Comparison of total PGE₂ levels at different stages of mammary gland development using the Tukey-Kramer HSD multiple comparison test revealed that increases in PGE₂ levels during lactation were significantly different ($P < 0.05$) from those detected during all pregnancy stages. Consistent with this, high levels of PGE₂ were also measured in breast milk expressed from lactating mammary glands (data not shown). Because the biosynthesis of PGE₂ is regulated by a number of enzymes, we next examined the steady-state RNA levels of COX-1, COX-2, and all three terminal PGE synthases at different developmental stages (Fig. 1B). Expression levels at each developmental stage were normalized to GAPDH (internal reference) and compared with those of the resting virgin gland (Fig. 1C). While expression of COX-2 could not be detected at any of the stages by Northern blot analysis (data not shown). The expression of $dPGES$ increased during pregnancy, reaching maximal levels by late pregnancy (Fig. 1C, day 16 pregnancy), and returned to levels comparable to those of the virgin gland by day 10 of involution. Expression levels of $EP1$, although barely detectable in the virgin gland, increased nearly 200-fold by late gestation (Fig. 2B, day 16 pregnancy), remained high during lactation, and returned to levels comparable to those of the virgin gland by day 10 of involution. Expression levels of $EP4$, although much lower than those of $EP2$, also increased dramatically by day 16 of gestation. Similar to $EP2$, $EP4$ RNA levels remained higher in the lactating gland and returned to levels comparable to those of the resting virgin gland by the completion of involution (Fig. 2B). Expression of $EP1$ was further analyzed by real-time quantitative PCR. In accordance with the Northern blot experiments, $EP1$ mRNA levels, although readily detected in the kidney, were extremely low and barely detectable at all stages of mammary development (data not shown). Because the maximal expression of $EP2$ preceded peak PGE₂ levels detected in the mammary gland, we investigated the possibility that $EP2$ may regulate PGE₂ synthesis in an autocrine manner. PGE₂ levels, therefore, were measured in mammary glands of $EP2^{-/-}$ mice at the developmental stages described above. PGE₂ levels were found to be comparable to those of wild-type mice in virgin $EP2^{-/-}$ mammary glands. Furthermore, no significant changes in PGE₂ levels were detected in mammary glands of $EP2^{-/-}$ mice compared with their wild-type counterparts at day 17 of pregnancy, when $EP2$ expression would be maximal, or on day 4 of lactation (Fig. 2C).

COX-1 is necessary for PGE₂ biosynthesis in the normal mammary gland

Because the increase in COX-1 RNA by day 5 of lactation paralleled the increase in PGE₂ levels during lactation, we examined COX-1 protein expression levels in greater detail at all developmental time points. COX-1 protein levels increased dramatically by day 1 of lactation (Fig. 3A), remained high on day 4 of lactation, similar to the steady-state RNA levels, and returned to levels comparable to those of the virgin gland by day 10 of involution. On longer exposure of the Western blot, low levels of COX-1 expression were also detected on days 9 and 14 of pregnancy and on day 10 of involution (data not shown). Therefore, we measured PGE₂ levels in lactating mammary glands derived from $COX-1^{+/+}$, $COX-1^{-/-}$, and $COX-1^{+/+}$ mice. PGE₂ was almost undetectable in mammary glands of $COX-1^{-/-}$ lactating glands ($P = 0.007$, $COX-1^{+/+}$ vs. $COX-1^{-/-}$, by Student’s $t$-test) and in breast milk ex-

Expression of the PGE₂ receptors EP2 and EP4 is maximally induced during pregnancy in the normal mammary gland

The intracellular effects of PGE₂ are mediated by four receptors, EP1–EP4, which activate diverse signaling pathways, potentially regulating multiple biological functions. Additionally, these receptors have unique tissue expression profiles. To facilitate the understanding of PGE₂ functions in mammary tissue homeostasis, we also examined the expression of all receptor isoforms during normal mammary gland development. The steady-state levels of $EP1$, $EP2$, $EP3$, and $EP4$ RNA were analyzed by Northern blotting (Fig. 2A). Expression levels were normalized to GAPDH and compared with those of the resting virgin gland (Fig. 2B). $EP3$ expression was high in the virgin gland and through the early and mid pregnancy stages. $EP3$ RNA levels were reduced dramatically by day 16 of pregnancy and in day 5 lactating tissue. $EP1$ expression, in contrast, was not detectable by Northern blotting at any developmental stage. The expression of $EP2$, although barely detectable in the virgin gland, increased nearly 200-fold by late gestation (Fig. 2B, day 16 pregnancy), remained high during lactation, and returned to levels comparable to those of the virgin gland by day 10 of involution. Expression levels of $EP4$, although much lower than those of $EP2$, also increased dramatically by day 16 of gestation. Similar to $EP2$, $EP4$ RNA levels remained higher in the lactating gland and returned to levels comparable to those of the resting virgin gland by the completion of involution (Fig. 2B). Expression of $EP1$ was further analyzed by real-time quantitative PCR. In accordance with the Northern blot experiments, $EP1$ mRNA levels, although readily detected in the kidney, were extremely low and barely detectable at all stages of mammary development (data not shown). Because the maximal expression of $EP2$ preceded peak PGE₂ levels detected in the mammary gland, we investigated the possibility that $EP2$ may regulate PGE₂ synthesis in an autocrine manner. PGE₂ levels, therefore, were measured in mammary glands of $EP2^{-/-}$ mice at the developmental stages described above. PGE₂ levels were found to be comparable to those of wild-type mice in virgin $EP2^{-/-}$ mammary glands. Furthermore, no significant changes in PGE₂ levels were detected in mammary glands of $EP2^{-/-}$ mice compared with their wild-type counterparts at day 17 of pregnancy, when $EP2$ expression would be maximal, or on day 4 of lactation (Fig. 2C).

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pressed from COX-1−/− lactating females compared with their wild-type littermates (Fig. 3C). Interestingly, PGE2 levels were also reduced significantly in COX-1−/−/H11001/H11002/lactating females compared with their wild-type littermates (Fig. 3C). Interestingly, PGE 2 levels were also reduced significantly in COX-1−/−/H11001/H11002 glands compared with wild-type controls (P = 0.014, COX-1−/−/H11001 vs. COX-1−/−/H11002, by Student’s t-test) (Fig. 3B). Concordantly, intermediate COX-1 protein levels were detected in the mammary glands of lactating COX-1−/−/H11002 females compared with wild-type females (Fig. 3B). Whole-mount and histological analyses of COX-1−/−/H11002 mammary glands did not reveal any abnormalities compared with wild-type controls (data not shown). To verify that loss of COX-1 did not alter the expression profiles of other PGE synthases, we analyzed the expression of mPGES1 and mPGES2 in COX-1−/−/H11002 glands on day 4 of lactation by real-time quantitative PCR. mPGES1 levels were increased slightly in COX-1−/−/H11002 lactating glands compared with COX-1−/−/H11001 mice, but this difference was not statistically significant (Fig. 3D). Similarly, mPGES2 and cPGES expression levels (data not shown) were not significantly different between COX-1−/−/H11002 and COX-1−/−/H11001 lactating mammary tissues.

**PGE2 synthesis in vivo is mediated by mPGES1 and COX-1 coupling**

As described above, the PGE2 synthases mPGES1, mPGES2, and cPGES are expressed at varying levels in the mammary gland, with mPGES2 and cPGES RNA levels increasing by mid pregnancy. However, the expression profiles of these synthases do not exactly coincide with that of COX-1 or with the total PGE2 levels measured, particularly during lactation. Although in vitro studies have demonstrated that cPGES couples preferentially with COX-1, mPGES1 is thought to be the primary partner for COX-2-
directed PGE2 biosynthesis. In contrast, mPGES2 was demonstrated to cooperate with either COX-1 or COX-2 in vivo. To clearly define the COX-1 coupling selectivity of PGE2 synthases in mammary tissue in vivo, we measured PGE2 levels in lactating mammary glands of mPGES1/−/− mice. As shown in Fig. 4A, PGE2 levels are reduced significantly in mPGES1/−/− mice compared with their wild-type counterparts (P = 0.0053, mPGES1+/+ vs. mPGES1/−/−, by Student’s t-test). To confirm that the decrease in PGE2 levels measured in mPGES1/−/− mammary glands was not attributable to alterations in the expression levels of either the COX genes or other terminal PGE synthases, we next measured the expression levels of COX-1, COX-2, and all PGE synthases in lactating mammary glands of mPGES1/−/− mice by real-time quantitative PCR (Fig. 4B). As described previously, extremely low COX-2 expression levels were detected in both mPGES1+/+ and mPGES1/−/− lactating glands (data not shown). COX-1 levels were identical in both mPGES1 wild-type and mPGES1/−/− lactating mammary glands, as expected. mPGES2 expression levels were not significantly different in mPGES1/−/− glands compared with those in wild-type controls. Although cPGES levels were slightly decreased in mPGES1/−/− lactating mammary glands, this change was not statistically significant compared with mPGES1+/+ mice. To further investigate the contribution of mPGES2 to PGE2 production in vivo, we
The mammary gland is a complex and dynamic organ composed of multiple tissue types. The physiology of the mammary gland is regulated by systemic factors like the ovarian-pituitary axis hormones, local growth factors, and products of various biochemical and metabolic pathways. Although the role of PGE_{2} and enzymes of this biosynthetic pathway, particularly the COXs, is being extensively studied, little is known about their functions in normal mammary gland tissue homeostasis. The postnatal development of the adult murine mammary gland has been completely established. PGE_{2} levels in the mammary gland are shown in Fig. 5C. PGE_{2} levels were decreased slightly in mPGES2/−/− mice but not significantly different from those detected in wild-type controls (P = 0.5, mPGES2/+/+ vs. mPGES2/−/−, by Student’s t-test). More interestingly, PGE_{2} production was not reduced to levels comparable to those measured in mPGES1/−/− or COX-1/−/− mice (Figs. 3A, 4A). To further verify that PGE_{2} production during lactation was mediated primarily by mPGES1, we measured levels of PGE_{2} and several other prostanoids by LC-MS in lactating mammary glands of mPGES1+/+, mPGES1/−/−, and mPGES1/−/− mice. Representative chromatograms for the PGE_{2} standard (Fig. 5A), a high PGE_{2} sample from a mPGES1/−/− mammary gland (Fig. 5B), and a low PGE_{2} sample from a mPGES1/−/− gland are shown in Fig. 5C. PGE_{2} levels were not significantly different between mPGES1+/+ and mPGES1/−/− mice, although a nearly 50% reduction in PGE_{2} was measured in the mPGES1/−/− mice. PGE_{2} levels were decreased significantly in mammary glands of mPGES1/−/− mice compared with mPGES1+/+ mice (P = 0.036) (Fig. 5D). Although mean PGE_{2} levels were decreased substantially (nearly 10-fold) in mPGES1/−/− glands compared with wild-type controls, this difference did not reach statistical significance because of the high variability in the levels of PGE_{2} measured in mPGES1/−/− mice. No significant differences in the levels of 6-keto-PGF_{1α} and PGF_{2} were measured in mPGES1/−/− mammary glands compared with either mPGES1+/+ or mPGES1/−/− mice (data not shown). Interestingly, PGD_{2} levels, which were extremely low and largely below the threshold of detection in mPGES1+/+ and mPGES1/−/− mice, increased nearly 3- to 4-fold in mPGES1/−/− samples. A similar trend was also observed for the levels of TXB_{2} measured in mPGES1/−/− lactating mammary glands (data not shown).

**DISCUSSION**

measured PGE_{2} levels in day 4 lactating mammary glands of mPGES2/−/− mice, recently generated in our laboratory (L. Jania, L. P. Audoly, and B. H. Koller, unpublished data). PGE_{2} levels were decreased slightly in mPGES2/−/− mice but not significantly different from those detected in wild-type controls (P = 0.5, mPGES2/+/+ vs. mPGES2/−/−, by Student’s t-test). More interestingly, PGE_{2} production was not reduced to levels comparable to those measured in mPGES1/−/− or COX-1/−/− mice (Figs. 3A, 4A). To further verify that PGE_{2} production during lactation was mediated primarily by mPGES1, we measured levels of PGE_{2} and several other prostanoids by LC-MS in lactating mammary glands of mPGES1+/+, mPGES1/−/−, and mPGES1/−/− mice. Representative chromatograms for the PGE_{2} standard (Fig. 5A), a high PGE_{2} sample from a mPGES1/−/− mammary gland (Fig. 5B), and a low PGE_{2} sample from a mPGES1/−/− gland are shown in Fig. 5C. PGE_{2} levels were not significantly different between mPGES1+/+ and mPGES1/−/− mice, although a nearly 50% reduction in PGE_{2} was measured in the mPGES1/−/− mice. PGE_{2} levels were decreased significantly in mammary glands of mPGES1/−/− mice compared with mPGES1+/+ mice (P = 0.036) (Fig. 5D). Although mean PGE_{2} levels were decreased substantially (nearly 10-fold) in mPGES1/−/− glands compared with wild-type controls, this difference did not reach statistical significance because of the high variability in the levels of PGE_{2} measured in mPGES1/−/− mice. No significant differences in the levels of 6-keto-PGF_{1α} and PGF_{2} were measured in mPGES1/−/− mammary glands compared with either mPGES1+/+ or mPGES1/−/− mice (data not shown). Interestingly, PGD_{2} levels, which were extremely low and largely below the threshold of detection in mPGES1+/+ and mPGES1/−/− mice, increased nearly 3- to 4-fold in mPGES1/−/− samples. A similar trend was also observed for the levels of TXB_{2} measured in mPGES1/−/− lactating mammary glands (data not shown).
crease modestly during pregnancy, which is marked by high epithelial proliferation, but increase dramatically during lactation (Fig. 1A). Thus, high PGE2 levels appear to be associated with terminal differentiation, rather than proliferation of mammary epithelium during normal gland development. These findings are also consistent with those previously reported for the rodent mammary gland (21), in which PGE2 levels were found to be lower in the resting virgin gland and highest in lactating mammary glands.

PGE2 metabolic enzymes are reported to have tissue-specific expression patterns and are putatively regulated by specific stimuli and cellular stresses. To identify the primary enzymes modulating PGE2 concentrations in the normal mammary gland, we examined the steady-state RNA levels of all the genes implicated in PGE2 biosynthesis and catabolism at different developmental stages. COX-1 RNA was extremely low in the virgin gland, but levels increased nearly 6-fold during lactation. In contrast, COX-2 expression levels in normal mammary tissue were not detectable by Northern blotting in our experiments. However, COX-2 expression was detected at very low levels at all developmental time points by real-time quantitative RT-PCR. A 3-fold increase in COX-2 mRNA levels measured in the involuting gland (Fig. 1C) possibly reflects the contribution of infiltrating macrophages and neutrophils activated by massive tissue remodeling and inflammation during mammary gland regression (22). Similar to COX-1, mPGES1 expression was easily detected by Northern blotting at all mammary developmental time points. However, unlike COX-1, mPGES1 levels varied little during preg-

Fig. 5. PGE2 levels were measured in inguinal mammary glands of mPGES1+/+ (n = 5), mPGES1+/− (n = 4), and mPGES1−/− (n = 4) animals on day 4 of lactation by liquid chromatography-mass spectrometry. A: Representative chromatogram for the PGE2 standard (10 ng/ml) in which PGE2 has the peak with 6.49 retention time. (PGD2 has a retention time of 6.75 in this chromatogram.) B: Representative chromatogram for a high-PGE2 sample (mPGES1+/+) based on the 6.4 retention time peak. C: Representative chromatogram for a low-PGE2 (mPGES1−/−) sample. D: PGE2 levels are presented as means ± SEM. * Statistically significant (P < 0.05) difference in PGE2 levels between mPGES1+/+ and mPGES1−/− mice. The lower limit of quantitation for PGE2 is 0.0902 ng/mg protein and is indicated by the dotted line.
nancy or lactation. The two putative synthases, \( mPGES2 \) and \( dPGES \) are also highly expressed in the mammary gland. In contrast to \( mPGES1 \), \( cPGES \) and \( mPGES2 \) levels are increased at mid pregnancy (days 14–16 of pregnancy) and lactation compared with the resting virgin gland. \( PGE_2 \) is rapidly catabolized to a less active 15-keto metabolite by \( PGDH \). We show for the first time that \( PGDH \) is expressed in the mammary gland at most developmental stages, suggesting that \( PGE_2 \) concentrations can be locally modulated in mammary tissue. Interestingly, we were unable to detect any \( PGDH \) RNA during lactation. The downregulation of \( PGDH \) may thus contribute to the maintenance of high \( PGE_2 \) levels in the lactating mammary gland.

The intracellular effects of \( PGE_2 \) are mediated by four receptors, \( EP1 \)–\( EP4 \), which have widely varying tissue distributions. They act through different G-proteins and signaling intermediates, thus modulating multiple and sometimes opposing cellular functions in a tissue. \( EP2 \) and \( EP4 \) are coupled to \( G_s \) and when activated cause increases in intracellular \( Ca^{2+} \) levels. \( EP3 \) is coupled to both \( G_s \) and \( G_q \) and upon activation increases intracellular \( Ca^{2+} \) levels. It is still unclear which specific G-protein is coupled with the \( EP1 \) receptor, although activation of \( EP1 \) also results in increased intracellular \( Ca^{2+} \) levels (23). The diversity of receptor-mediated signaling further increases the complexity of defining the functions of \( PGE_2 \) in normal mammary gland homeostasis. Therefore, we analyzed the steady-state RNA levels of all \( EP \) genes during mammary development. We were unable to detect \( EP1 \) expression by Northern blotting, and \( EP1 \) mRNA levels were only barely above the threshold of detection, even by real-time quantitative PCR analysis, at all stages of development. \( EP3 \), however, was expressed highly at all mammary developmental stages except late pregnancy (day 16), when expression levels were significantly reduced. \( EP3 \) RNA levels were reduced to nearly half of those detected in the virgin gland at day 5 of lactation and returned to levels comparable to those of the virgin gland by day 10 of weaning. Interestingly, \( EP2 \) expression, which was undetectable in the virgin gland, returned to levels comparable to those of nearly half of those detected in the virgin gland at day 5 of lactation and remained high compared with the virgin gland. \( EP4 \) has an expression profile similar to \( EP2 \), although the RNA is expressed at much lower levels. This is in contrast to most tissues that coexpress these receptors, in which \( EP4 \) is usually detected at much higher levels (24, 25). It has been suggested that higher \( EP4 \) levels are maintained across most tissues in part because of the faster desensitization of \( EP4 \) compared with \( EP2 \) (26). Although the specific receptor activities of \( EP2 \) and \( EP4 \) still remain to be determined, in the mammary gland, \( EP2 \) would potentially predominate in mediating \( PGE_2 \) effects because of its higher expression levels and slower desensitization rates. Because maximal \( EP2 \) levels are detected before lactation and high expression levels are maintained during lactation, we explored the possibility that \( EP2 \) may regulate \( PGE_2 \) synthesis in an autocrine manner. Evidence for this feedback mechanism has been documented in \( EP2^{-/-} \) mice: \( COX-2 \) RNA levels were decreased signifi-
levels during lactation. Furthermore, PGE$_2$ was almost undetectable in COX-1$^{-/-}$ lactating mammary glands and in breast milk expressed from COX-1$^{-/-}$ lactating females compared with wild-type littermates. These data demonstrate that COX-1 is necessary for PGE$_2$ biosynthesis in the normal mammary gland. Although the expression of the recently identified COX-1b protein (also referred to as COX-3) in the murine mammary gland has not been determined, it remains possible that COX-1b, if expressed, could also contribute to PGE$_2$ biosynthesis during lactation. Because cPGES and mPGES2 expression levels were increased during mid pregnancy (Fig. 1C), we hypothesized that these synthases could partner with COX-1 for PGE$_2$ synthesis during lactation in vivo. cPGES has been demonstrated to work in concert with COX-1 for PGE$_2$ synthesis in vitro by coexpression of these proteins. It is unclear at present which COX isoform mPGES2 preferentially partners with in vivo, although in vitro studies suggest a modest preference for COX-2. mPGES1, on the other hand, is thought to partner specifically with COX-2 for delayed PGE$_2$ biosynthesis during inflammatory responses. To clarify these mechanisms, we first assessed PGE$_2$ levels in lactating mammary glands derived from mPGES1$^{-/-}$ and mPGES1$^{+/+}$ females. Surprisingly, in mPGES1$^{-/-}$ mice, PGE$_2$ levels were decreased significantly compared with both wild-type mice (Fig. 4A) and heterozygous mice (Fig. 5B). This decrease did not result from changes in expression levels of COX-1 (Fig. 4B) or from increases in the expression of the catabolic enzyme PGDH (data not shown). Together with the observation that COX-2 expression is barely detectable in the normal gland, these data strongly suggest that mPGES1 and COX-1 are primary functional partners in vivo for mammary PGE$_2$ biosynthesis, particularly during lactation. Evidence for such a partnership in vivo is growing. Studies have demonstrated that in the acetic acid-induced pain hypersensitivity model, mPGES1$^{-/-}$ mice have reduced writhing responses similar to those observed in COX-1$^{-/-}$ but not in COX-2$^{-/-}$ mice (10, 37). Additionally, mPGES1$^{-/-}$ macrophages are deficient in both acute and delayed PGE$_2$ biosynthesis, similar to defects observed in macrophages isolated from COX-1$^{-/-}$ and COX-2$^{-/-}$ mice, respectively. From these data, it was inferred that mPGES1 could pair with either COX isofrom (10), instead of being coupled exclusively with COX-2. A recent study demonstrating that mPGES1 and COX-1 cooperate for basal PGE$_2$ biosynthesis in the murine stomach (38) further supports our findings. These data suggest that a functional COX-1/mPGES1 pairing may be common in certain tissue types under normal physiological conditions.

Because PGE$_2$ levels in mPGES1$^{-/-}$ lactating glands are extremely reduced despite high expression levels of mPGES2 and cPGES, and because PGE$_2$ levels are not significantly different in mPGES2$^{-/-}$ lactating mammary glands (Fig. 4C) compared with wild-type mice, it appears that cPGES and mPGES2 do not contribute significantly to PGE$_2$ biosynthesis in mammary tissue. However, mean PGE$_2$ levels measured in mPGES1$^{-/-}$ mice (3.33 pg/mg tissue; Fig. 4A) remained higher than those detected in COX-1$^{-/-}$ mice (0.96 pg/mg tissue; Fig. 3B). It is difficult to ascertain the significance of this difference, because mPGES1$^{-/-}$ and COX-1$^{-/-}$ mice are maintained on different genetic backgrounds. The residual PGE$_2$ levels detected in mPGES1$^{-/-}$ mammary glands may result from experimental variability or reflect nonenzymatic formation of PGE$_2$ (39–41). Alternatively, it remains possible that cPGES may contribute to basal PGE$_2$ production in the mammary gland, potentially via a COX-1 partnership, especially because cPGES is expressed abundantly at all stages of mammary gland development. Analysis of mPGES1$^{-/-}$ mice, when available, will help define the primary COX partnership and determine the contribution of this enzyme to PGE$_2$ biosynthesis in the mammary gland. Although mPGES1$^{-/-}$ mice produced barely detectable PGE$_2$ levels during lactation, no increases in mPGES1 RNA levels were observed in wild-type mice at this stage (Fig. 1B, C). It is possible that changes in mPGES1 protein levels, enzymatic activity, or cellular compartmentalization may be responsible for increasing the efficiency of PGH$_2$ catalysis and PGE$_2$ production. Alternatively, the availability of substrate (i.e., the conversion of AA to PGH$_2$ by COX-1) may be the principal regulatory step. This is partly supported by the significant reduction of PGE$_2$ levels detected in lactating glands of COX-1$^{-/-}$ mice compared with COX-1$^{+/+}$ controls (Fig. 3B). LC-MS measurements of multiple eicosanoids in mPGES1$^{-/-}$ mammary glands revealed that PGD$_2$ levels were significantly increased in mPGES1$^{-/-}$ lactating mice compared with both wild-type and mPGES1$^{+/+}$ mice. Similarly, increased levels of TXB$_2$ were measured in mPGES1$^{-/-}$ mammary samples. Although it is difficult to interpret these data because PGD$_2$ and TXB$_2$ levels measured by LC-MS in mPGES1$^{+/+}$ and mPGES1$^{-/-}$ mammary glands are largely below the limit of quantitation, these observations are qualitatively similar to those reported recently by Boulet et al. (38). They propose that increased basal PGD$_2$ and TXB$_2$ levels in murine mPGES1$^{-/-}$ stomach potentially result from an increased shunting of excess unused PGH$_2$ substrate to alternative prostanooid pathways. A recent report also demonstrates that prostaglandin production is redirected in mPGES1$^{-/-}$ macrophages, resulting in the increased synthesis of TXB$_2$ and 6-keto-PGF$_1\alpha$ (42). However, it remains possible that the increased PGD$_2$ levels observed in mPGES1$^{-/-}$ glands simply result from the lack of specificity in detecting PGD$_2$.

Our study also suggests that transcription of PGE$_2$ metabolism genes and PGE$_2$ effectors is regulated tightly during normal mammary gland development, although, at present, very little is known about these mechanisms. Although COX-1 expression is constitutive in most tissues, it appears to be specifically regulated during murine mammary epithelial differentiation. It is unclear at this time whether the increases in COX-1 RNA and protein levels during lactation represent increases in transcription, changes in RNA stability, or increased translation. The murine COX-1 promoter is relatively uncharacterized, and one study suggests that the promoter has functional glucocorticoid response elements with weak responsiveness to progesterone (43–45). Therefore, we can speculate that
COX-1 transcription may be positively modulated by the activity of glucocorticoid receptor hormones in combination with progesterone during lactation. Information about the regulation of the murine PGDH promoter is currently limited (46). However, a recent study demonstrates that the human PGDH promoter is activated by PRs, PR-A and PR-B (47). Interestingly, PR RNA levels are higher during pregnancy and lowest during lactation, similar to the PGDH expression profile in the murine mammary gland (48). It is possible, therefore, that PGDH transcription is positively regulated by progesterone. Consequently, PGDH expression would be low when PR levels are decreased, thus allowing high amounts of PGE2 to be maintained during lactation. The temporal expression profiles of EP2 and EP4 suggest that ovarian hormones, specifically estrogen and progesterone, may directly or indirectly regulate these genes. Expression of EP2 and EP4 is regulated by ovarian estrogen and progesterone during implantation in the luminal epithelium of the uterus (49, 50). Additionally, novel PR binding sites have been identified recently in the murine EP2 promoter (51), suggesting that EP2 may be differentially regulated in the mammary gland by progesterone.

In conclusion, our study demonstrates that PGE2 metabolism is temporally regulated during normal murine mammary gland development. PGE2 production in the murine mammary gland appears to be dependent on the functional pairing of COX-1 and mPGES1, and this report demonstrates for the first time that mPGES1 is the primary synthase mediating PGE2 biosynthesis during normal mammary homeostasis. PGE2 effectors, particularly the G-coupled receptors EP2 and EP4, are also regulated in a temporal manner. Furthermore, the disconnection between points of maximal PGE2 synthesis and receptor expression levels suggests that PGE2 may perform distinct functions at different stages of mammary development. PGE2 via the actions of EP2/EP4 may regulate mammary-specific cellular functions during pregnancy, whereas high amounts of PGE2 secreted into breast milk may modulate infant physiology or immune responses (52, 53). Specifically, PGE2 may promote intestinal maturation or motility in the infant (54, 55) or be cytoprotective during postnatal development of the intestinal mucosa. Identifying specific mammary cell populations that express the different PGE synthases and receptors, coupled with the analysis of mammary epithelial proliferation and apoptosis in receptor and mPGESI−/− mice, will help define the normal physiological functions of PGE2 in the murine mammary gland.

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