PTGS2 (Prostaglandin Endoperoxide Synthase-2) Expression in Term Human Amnion *in Vivo* Involves Rapid mRNA Turnover, Polymerase-II 5′-Pausing, and Glucocorticoid Transrepression

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The *in vivo* role of glucocorticoids in controlling prostaglandin endoperoxide synthase-2 (PTGS2) expression in the human amnion is unclear despite extensive studies using *in vitro* models. We addressed this issue by determining PTGS2 mRNA levels and gene transcriptional activity, RNA polymerase-II (pol-II) binding, pol-II C-terminal domain (CTD) phosphorylation, histone acetylation, and histone methylation at the *PTGS2* gene in fresh amnion and in amnion explants incubated with dexamethasone for 24 h after delivery, when adaptation from *in vivo* to *in vitro* conditions occurred. PTGS2 mRNA turnover changed during incubation involving the initial rapid decrease and subsequent rebound of the transcription rate and stabilization of mRNA. pol-II accumulated in the 5′-region of the gene, which indicated postinitiation pausing. pol-II binding, 5′-accumulation, C-terminal domain Ser-5 and Ser-2 phosphorylation, and histone acetylation decreased rapidly and did not reverse during the transcriptional rebound, suggesting that the transcriptional mechanism altered *in vitro*. Dexamethasone decreased *PTGS2* gene activity and mRNA levels. Glucocorticoid receptor-α (GRα) was bound to the *PTGS2* promoter but did not affect pol-II recruitment, pausing, or the epigenetic marks. GRα binding, however, decreased initiating (Ser-5) and elongating (Ser-2) pol-II phosphorylation. The ability of the *PTGS2* promoter to bind GRα in response to dexamethasone diminished during incubation. We conclude that PTGS2 mRNA turnover is accelerated *in vivo*, but the underlying mechanisms are not sustained beyond 24 h in explants. Glucocorticoids chronically transrepress *PTGS2* gene activity *in vivo* in part by interfering with transcription initiation and elongation. Glucocorticoid transrepression of *PTGS2* may be important for pregnancy maintenance and the timing of parturition. **(*Endocrinology* 152: 2113–2122, 2011)**

The amnion membrane is a major source of prostaglandins (PG) in the pregnant human uterus (1). Both isoenzymes of prostaglandin endoperoxide synthase, PTGS1 and PTGS2 (also known as PGHS-1 and -2 or cyclooxygenase-1 and -2) are expressed in this tissue catalyzing the limiting irreversible step of PG biosynthesis (2, 3). PTGS2 expression increases with advancing gestation and becomes dominant over PTGS1 at term, contributing to the production of PG that are critically important for the initiation and maintenance of labor (4, 5). We have reported previously that this up-regulation is mainly transcriptional, but evidence suggested that posttranscriptional processes were also involved in the increase of steady-state PTGS2 mRNA levels at late gestation (6). The mechanisms underlying these changes are unclear and are part of the generally unknown mechanism controlling gestational length and the timing of parturition.

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Abbreviations: ChIP, Chromatin immunoprecipitation; CTD, C-terminal domain; hnRNA, heterogenous nuclear RNA; NFκB, nuclear factor-κB; pol-II, polymerase-II; PG, prostaglandin; PTGS2, prostaglandin endoperoxide synthase-2; qPCR, quantitative real-time PCR; qRT-PCR, quantitative real-time RT-PCR.

Glucocorticoids stimulate PGE_2 production and PTGS2 expression in primary cultures of amnion cells (7–10). Cortisol concentration increases sharply in amniotic fluid at term (11), suggesting that glucocorticoids may drive intrauterine PTGS2 levels and PG production eventually triggering labor (12). It has also been reported, however, that the stimulatory effect of glucocorticoids appears after 3 or 4 d of culture, and freshly isolated amnion cells and amnion tissue respond to glucocorticoid with a decrease in PG output (13–16). Moreover, experiments with primary cultures enriched in either epithelial or mesenchymal (fibroblastic) amnion cell types indicated that only the mesenchymal cells responded to glucocorticoid with PTGS2 induction, and in epithelial cells the steroid was inhibitory or ineffective (15, 17, 18). Recent work has shown that cortisol induces PTGS2 transcription in amnion fibroblast cultures by stimulating the phosphorylation and binding of cAMP response element-binding protein 1 to the cAMP response element in the proximal *PTGS2* promoter (19). Based on the cell specificity of stimulation, it was suggested that the overall stimulatory effect of glucocorticoid was an artifact and simply due to fibroblastic overgrowth after a few days of culturing mixed primary amnion cell populations (15). Conflicting data showing glucocorticoid stimulation of PG output from epithelial amnion cells within 72 h of culture have also been reported (20).

The uncertainty surrounding the action of glucocorticoids on amnion PG production impedes the understanding of the role of glucocorticoids in human parturition. In the present investigation, we sought to clarify this issue by determining *PTGS2* gene activity and mRNA levels in freshly delivered amnion and in amnion tissue explants exposed to glucocorticoid *in vitro* during the first 24 h after delivery. Within this period, PTGS2 expression undergoes a transition from the *in vivo* steady state to a state adapted to the *in vitro* incubation conditions (21). Furthermore, the cellular composition of freshly isolated tissue allows an assessment of the overall response of amnion to corticosteroids. Examining the transitional changes may differentiate regulatory mechanisms operating *in vivo* from adaptive phenomena elicited by the artificial *in vitro* environment.

Glucocorticoids can stimulate or inhibit gene expression through a variety of transcriptional, posttranscriptional, and epigenetic mechanisms (22). To delineate transcriptional regulation, we have measured glucocorticoid receptor (GRα) binding to the *PTGS2* promoter and RNA polymerase II (pol-II) distribution along the *PTGS2* gene. Serine-5 (Ser-5) and Serine-2 (Ser-2) phosphorylation of the pol-II large subunit C-terminal domain (CTD) tail heptapeptides is linked to transcriptional initiation and elongation, respectively (23). Therefore, we have determined the Ser-5 and Ser-2 phosphorylation status of pol-II associated with the *PTGS2* gene and assessed glucocorticoid-induced changes at the transcription initiation and elongation phases. In addition, histone-3 and -4 acetylation and histone-3-lysine-4 methylation at the *PTGS2* promoter were determined to explore the contribution of epigenetic modifications to the glucocorticoid-dependent regulation of the *PTGS2* gene.

Our results suggest that glucocorticoids negatively regulate PTGS2 expression in the amnion and exert a chronic inhibitory action *in vivo* at the transcriptional and possibly the posttranscriptional levels. This is in agreement with the clinical observation that glucocorticoids do not induce labor in women. Moreover, our results demonstrate that the transcriptional apparatus controlling *PTGS2* gene activity in amnion reorganizes spontaneously within 24 h *in vitro*, and its ability to interact with the GRα-glucocorticoid complex diminishes. These rapid changes call for extreme caution when *PTGS2* gene expression information obtained in amnion culture models beyond this time is extrapolated to the *in vivo* functioning of the gene.

Materials and Methods

Materials

Affinity-purified rabbit polyclonal antibodies to GR-α (sc1002) and pol-II (sc899), Protein A-agarose, rabbit IgG, and mouse IgM were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Chromatin immunoprecipitation (ChIP) grade antihistone-3 (dimethyl K4) antibody (H3K4me2, ab 7766) was from Abcam Inc. (Cambridge, MA). Anti-acetyl-Histone 3 (acH3, 06-599) and anti-acetyl-histone-4 (acH4, 06-598) antibodies were from Millipore Corp. (Billerica, MA). Mouse monoclonal IgM against pol-II phosphorylated at the CTD serine-2 (H5) and serine-5 (H14), respectively, were purchased from Covance Laboratories, Inc. (Princeton, NJ). Rabbit antimouse IgM (μ-specific, SAB1171) was from Open Biosystems (Huntsville, AL). Ethylene glycol-bis [succinimidylsuccinate] was from Pierce Chemical Co. (Rockford, IL). Rnase-free Dnase and RNeasy mini kits were from QIAGEN (Hilden, Germany). DMEM:F12 medium supplemented with 15 mM HEPES and L-glutamine, TRIzol reagent, Superscript III First Strand Synthesis kits, and PCR primers were supplied by Invitrogen (Carlsbad, CA). Alien quantitative real-time RT-PCR (qRT-PCR) Inhibitor alert kit was from Stratagene (La Jolla, CA). L-Glutathione (reduced), BSA fraction V (BSA, Sigma no. A 9647), dexamethasone, and other chemicals were from Sigma-Aldrich (St Louis, MO).

Patients and tissue collection

Fetal membranes were collected from women undergoing elective cesarean section at term (37–41 wk gestation) in the absence of labor. Previous cesarean section was the indication in all cases. Reflected amnion was separated from the chorio-decidua within 30 min of delivery and processed immediately for RNA extraction, ChIP, or tissue incubation as required.

Informed consent to donate tissues was obtained from women at the John Hunter Hospital in Newcastle, Australia, under approval from the Hunter New England Health Human Ethics Committee and the University of Newcastle Human Ethics Committee. Women with a history of infection, treated with nonsteroidal antiinflammatory drugs, diagnosed with chorioamnionitis or severe asthma were excluded from the study.

Amnion tissue incubation

Amnion membranes were cut into 1–2 cm^2 pieces and randomly grouped to reduce the impact of regional variability, as described elsewhere (21). Approximately 0.5 g of tissue was incubated at 37 C in 25 ml DMEM:F12 medium, supplemented with 1 mg/liter reduced L-glutathione, 0.01% BSA, and 40 μg/ml gentamicin. The incubations lasted for a maximum of 24 h, during which time the tissue explants remained viable as assessed by a lactate dehydrogenase release assay described previously (21). Dexamethasone was added to the medium in dimethylsulfoxide at a final vehicle concentration of 0.1% (vol/vol). After incubation the tissues were removed from the medium, blotted, frozen in liquid nitrogen, and stored at −80 C for RNA extraction, or processed immediately for ChIP. The amnion incubation experiments were repeated four times using tissue from different women (n = 4).

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The RNA was purified using RNAse-free DNAse and RNeasy (QIAGEN) mini kits. RNA integrity was confirmed by the examination of 18S and 28s band formation in agarose gels. The purified RNA was spiked with Alien RNA (10^7 copies/μg) and converted to cDNA with Superscript III First Strand Synthesis kits, using random hexamers. Alien RNA was used as a spiked-in internal reference RNA (24) for PTGS2 mRNA, PTGS2 heterogenous nuclear RNA (hnRNA), and GR mRNA abundance determination in the incubation experiments by quantitative real-time RT-PCR (qRT-PCR).

ChIP

ChIP was performed as described elsewhere (25), with the modification of employing a dual cross-linking procedure (26). Briefly, 1 g of tissue was rinsed in PBS, blotted, and placed in 20 ml of PBS containing 2 mM ethylene glycol-bis [succinimidylsuccinate], a protein-protein cross-linking reagent, dissolved immediately before use in 50 μl dimethylsulfoxide. After a 30-min incubation at room temperature, the tissue was rinsed twice in PBS and placed in 1% formaldehyde as the second cross-linking agent, and the normal ChIP protocol (25) was continued.

Antibody concentrations per 0.5 ml immunoprecipitation mixture, optimized in preliminary experiments, were: 3 μg for GRα, pol-II (sc899), and acH3; 5 μg for acH4; 2 μg for H3K4me2; 8–12 μg for ser-2-phosphorylated pol-II (H5); and 3–5 μg for ser-5 phosphorylated pol-II (H14). To reduce nonspecific binding, immunoprecipitations and subsequent washings were performed in low DNA-binding microcentrifuge tubes (Eppendorf, North Ryde, Australia), and the protein A-agarose used for capture of the immune complexes was blocked overnight with 0.5 ml Buffer-3 (25) containing 50 μg BSA and 2 μg salmon sperm DNA.

The mouse phospho-specific RNA pol-II antibodies (H5 and H14) and control IgM required an additional step, because protein A-agarose does not bind IgM class antibodies. To overcome this problem, rabbit antimouse IgM (μ-specific, 5–12 μg) secondary antibody was preincubated with protein A-agarose for 1.5–2 h. Excess secondary antibody was removed by washing with Buffer-3, and the sample was incubated with the protein A-agarose for 2 h at 4 C to capture the IgM complexes. The ChIP procedure was then continued as previously described including washings, cross-link reversal, and DNA purification (25). In all ChIP protocols, antibodies were replaced with IgG or IgM as the negative control.

GRα and H3K4me2 distribution along the *PTGS2* gene was determined in six and three independent ChIP experiments, respectively. Phospho-specific pol-II binding (with H5 and H14 antibodies), histone-3 acetylation, and dexamethasone-dependent GRα binding were determined in four experiments, whereas total pol-II (using the sc899 antibody) binding and histone 4 acetylation were determined in three independent ChIP experiments using tissue explants from different individuals.

Quantitative real-time PCR (qPCR)

qPCR was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Scoresby, Victoria, Australia). Each amplification was performed in triplicate in reaction mixtures containing SYBR Green PCR master mix (Applied Biosystems), primer, and template to a total volume of 25 μl. The primer sequences, positions, and concentrations for PTGS2 mRNA, and the dominant GRα mRNA variant determinations were described in Ref. 21 and Ref. 27, respectively, and are listed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. Primers flanking the *PTGS2* TATA-box site and seven additional sites (R2–R8) distributed along a 2579-base upstream promoter region were described by Mitchell *et al.* (25). The primer sequences used for PTGS2 hnRNA determinations amplify a region spanning the junction of exon 4 and intron 4, whereas the seven downstream primer pairs (E1–E5, E7, and E8) are located in each of the exons 1–5, 7, and 8 on the *PTGS2* gene, as listed in Supplemental Table 1. Primer Express software could not find a region suitable for primer placement in the short exon 6. The Alien reference primer sequences are proprietary, and these primers were used as per the manufacturer's instructions. The remaining primer concentrations were 200 nM for the TATA site, 600 nM for exon 2, and 400 nM for the other exon sites. No-template controls and no-reverse transcriptase controls were included in each PCR run as appropriate. Template amounts were optimized, and amplification efficiency was tested as described elsewhere (28) and was routinely confirmed using LinReg PCR software (29). Dissociation curve analysis was used to determine homogeneity of the PCR products.

Data analysis

qRT-PCR and qPCR data were analyzed using the $\Delta\Delta C_T$ method (28). PTGS2 mRNA, PTGS2 hnRNA, and GRα mRNA abundance values were determined relative to the constant amount of spiked-in Alien RNA reference (Stratagene). ChIP data determining the distribution of pol-II, GRα, H3K4me2, acH3, and acH4 along the *PTGS2* gene were processed relative to input and normalized to an upstream promoter site (R8). Promoter binding of GRα was calculated as per cent recovery of input over the IgG negative control. Data were tested for distribution, transformed to approximate normal distribution and

homogeneity of variance, and subjected to ANOVA (with repeated measures). Pairwise comparisons were performed using paired *t* tests. In all statistical analyses, $P < 0.05$ was considered significant. Analyses were carried out using STATA version 8.0 (College Station, TX). IC_{50} values were calculated using GraphPad Prism Software (La Jolla, CA).

Results

PTGS2 mRNA level and gene activity; effect of glucocorticoid

Amnion tissue was isolated within 30 min after elective cesarean delivery and incubated for 24 h in a chemically defined serum free medium (DMEM:F12). PTGS2 mRNA levels, shown in Fig. 1A, did not change during the first 4 h and increased several fold between 4 h and 24 h. In the presence of the synthetic glucocorticoid dexamethasone PTGS2 mRNA abundance did not increase and was significantly lower than in the vehicle-treated tissue both at 4 h and 24 h. PTGS2 hnRNA levels are presented in Fig. 1B under the same conditions. PTGS2 hnRNA is the precursor of the mature PTGS2 mRNA. and its abundance is a validated measure of the transcriptional activity of the *PTGS2* gene in amnion tissue (21). PTGS2 hnRNA levels fell precipitously *in vitro* reaching a nadir of about 15% by 4 h compared with fresh (0 h) tissue, and then rebounded to about 25% of the 0 h value by 24 h ($t_3 = 10.87$; $P < 0.002$). Dexamethasone augmented the fall of gene activity and abolished the rebound, resulting in a significant decrease in hnRNA levels both at 4 h and 24 h. The inhibitory effects of dexamethasone were concentration dependent as demonstrated in Fig. 2, A and B, with IC_{50} values of 2.8 ± 1.2 nM for PTGS2 mRNA and 4.6 ± 1.4 nM for PTGS2 hnRNA (mean ± SE, n = 4).

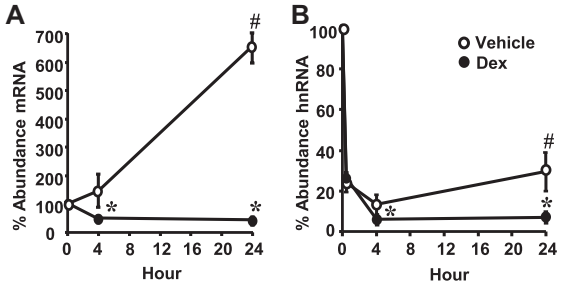

FIG. 1. Changes in PTGS2 mRNA levels and gene activity in amnion tissue *in vitro* and the effects of glucocorticoid. Amnion tissue samples were incubated with vehicle or dexamethasone (Dex, 100 nM) for the times indicated, and PTGS2 mRNA and hnRNA (a measure of gene activity) abundances were determined by qRT-PCR. Data are shown as per cent of the 0 h value (mean ± SE, n = 4 independent experiments). A, PTGS2 mRNA levels increased spontaneously by 24 h (#, $F_2 = 4.45$; $P < 0.03$, ANOVA), and dexamethasone inhibited the increase at both 4 h (*, $t_3 = -18.26$; $P < 0.001$); and 24 h (*, $t_3 = -17.95$; $P < 0.0011$, paired *t* test); B, PTGS2 hnRNA levels decreased, reaching a nadir at 4 h ($F_3 = 15.15$; $P < 0.001$) and rebounded between 4 h and 24 h (#, $t_3 = 10.87$; $P < 0.002$, paired *t* test). Dexamethasone caused a decrease of gene activity at 4 h compared with vehicle and prevented the rebound at 24 h (*, $t_3 = -4.94$; $P < 0.02$,) and 24 h (*, $t_3 = -10.82$; $P < 0.002$; paired *t* tests). Statistical analysis was performed with data transformed to approach normal distribution and homogeneity of variance.

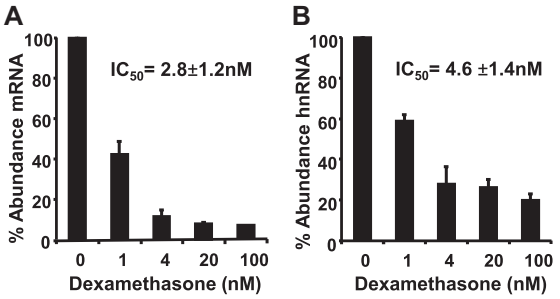

FIG. 2. The inhibition of PTGS2 mRNA levels and gene activity by dexamethasone is concentration dependent. Amnion tissue was incubated for 24 h with increasing concentrations of dexamethasone, and PTGS2 mRNA (A) and hnRNA (B) abundances were determined by qRT-PCR. Data are presented as per cent of the 0 h value (mean ± SE, n = 4).

GR binding to the *PTGS2* promoter

Repression of gene activity by glucocorticoids is often mediated by the interaction of the GR complex with the target gene promoters (22). We have examined this possibility by measuring GRα binding to the *PTGS2* promoter in freshly isolated amnion by ChIP. As shown in Fig. 3A, GRα binding was detected to the *PTGS2* proximal promoter region within the first 1000 bp upstream of the TATA site. Amplicon R2 (bases −248/−127 relative to the transcriptional start position) generated the strongest signal for GRα binding and was used subsequently to measure GRα binding to the *PTGS2* promoter in tissue explants. In the absence of dexamethasone, GRα binding decreased dramatically during incubation, and only minimal binding was seen at 4 h (Fig. 3B). There was a tendency for rebound between 4 h and 24 h, which did not reach statistical significance. In the presence of dexamethasone, GRα binding was maintained for 4 h at a level not different from the 0 h value. Binding had decreased by 24 h to a degree comparable to the vehicle-treated tissue despite the continuing presence of dexamethasone (Fig. 3B).

Loss of GRα expression during incubation may explain the decreasing GRα binding to the *PTGS2* promoter by 24 h. Furthermore, glucocorticoids can down-regulate their own receptor through a feedback mechanism (30). To assess these possibilities, we measured GRα mRNA abundance in our incubation system using qRT-PCR (Supplemental Fig. 1). GRα mRNA abundance did not change between 0 and 4 h and increased significantly between 4 h and 24 h of incubation. Dexamethasone prevented the spontaneous increase between 4 h and 24 h, but the GRα mRNA levels did not decrease below the 0 h value at any time during treatment with the glucocorticoid. This

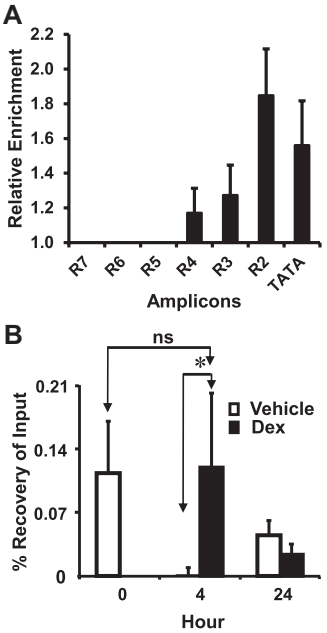

FIG. 3. GRα binding to the *PTGS2* promoter. A, ChIP was used to determine GRα binding to the upstream region of the *PTGS2* promoter between the TATA site (−62/+5) and amplicon R7 (−2253/−2203). Results are expressed relative to upstream site R8 (−2579/−2518; mean ± SE, n = 6). Amplicon positions are specified in Fig. 8B and Supplemental Table 1. GRα binding was maximal at R2. B, GRα binding to R2 in amnion incubated with vehicle or dexamethasone (Dex). Results are expressed as the mean ± SE (n = 4) of per cent recovery of input over background (IgG isotype control). GRα binding diminished almost completely by 4 h compared with fresh tissue (0 h) and did not increase significantly between 4 h and 24 h ($F_3 = 5.22$; $P < 0.03$). Dexamethasone maintained GRα binding to R2 at 4 h, but not at 24 h (*, $t_3 = -9.52$; $P < 0.0035$, paired *t* test). Statistical analysis was performed with transformed data.

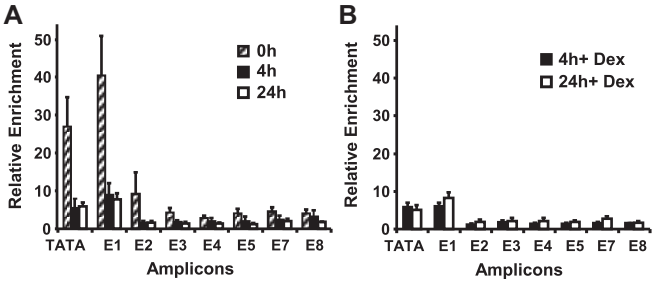

FIG. 4. RNA polymerase II binding to the *PTGS2* gene in amnion incubated with or without dexamethasone (Dex). ChIP was performed with an antibody directed to pol-II (sc899), and binding was analyzed between the TATA site and exon 8 (E8; see Fig. 8B and Supplemental Table 1 for positions). Relative enrichment was calculated as recovery of input relative to the upstream site R8 (mean ± SE, n = 3). The same scale is used on both panels to facilitate comparison. A, Pol-II accumulated in the TATA and E1 regions in fresh (0 h) and vehicle-treated (4 h and 24 h) tissues, but pol-II binding decreased between 0 h and 4 h ($F_2 = 15.97$; $P < 0.001$). B, Pol-II binding in the presence of dexamethasone. The steroid had no effect on the extent, time course, or distribution of pol-II binding compared with vehicle. Statistical analysis was performed with transformed data.

indicates that the reduced GRα binding to the *PTGS2* promoter at 24 h of incubation is not due to a loss of GRα expression despite some negative feedback effect by dexamethasone. Rather, it suggests that the ability of the *PTGS2* promoter to bind GRα in a glucocorticoid-dependent fashion diminished by 24 h of incubation.

RNA polymerase II binding and phosphorylation at the *PTGS2* gene

pol-II unphosphorylated at the large subunit CTD is recruited to promoters and is phosphorylated at specific serine residues when transcription is performed (31). CTD phosphorylation at Ser-5 is required for the initiation of transcription, and further phosphorylation at Ser-2 is needed for progressing to the elongation phase. The implication is that the distribution and CTD phosphorylation status of pol-II bound to a gene are informative about pol-II recruitment, transcription initiation, and elongation and can reveal regulation at these stages (23, 32, 33). We examined pol-II distribution along the *PTGS2* gene in amnion explants incubated in the presence and absence of dexamethasone. For this ChIP analysis, we used antibody sc899, which binds pol-II at the N-terminal domain regardless of its CTD phosphorylation state. Figure 8B shows the location of primer sites used for monitoring pol-II distribution between the TATA region and exon 8 (E8). The pol-II distribution in fresh tissue (0 h) showed a marked accumulation of the enzyme in the region of the TATA site and exon 1 (E1, Fig. 4A). This type of distribution is indicative of transcriptional pausing after pol-II recruitment and initiation (32, 33). Pol-II association with the gene decreased significantly across all sites by 4 h and stayed at this level at 24 h of incubation. Smaller, but significantly elevated pol-II levels at the 5′-region (TATA and E1) compared with downstream sites were still observable at both incubation times. Dexamethasone treatment did not change either the distribution or the extent of pol-II binding (Fig. 4B) compared with vehicle-treated tissue.

We have also determined the binding of Ser-5-phosphorylated (initiator) pol-II to the *PTGS2* gene by ChIP using an antibody (H14) specific for the Ser-5-phosphorylated CTD. The Ser-5-phosphorylated pol-II distribution in fresh tissue (0 h) exhibited a pattern similar to that of total pol-II (measured by sc899), indicating that the pol-II engaged in initiation, pausing, and elongation can all be phosphorylated at Ser-5 of the CTD (Fig. 5A). Further, initiator polymerase binding was significantly lower after 4 h of incubation than in fresh tissue (0 h) across all sites and did not change subsequently between 4 h and 24 h. Dexamethasone treatment (Fig. 5B) significantly decreased the level of CTD Ser-5 phosphorylation compared with vehicle ($t_{31} = 5.096$; $P < 0.001$, paired *t* test with data paired according to each amplicon and experiment). At

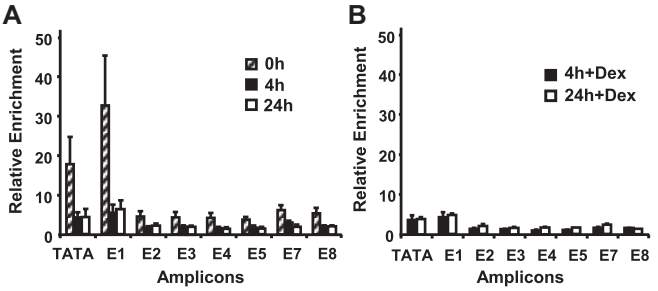

FIG. 5. Serine-5 (initiating) phosphorylation of Pol-II bound to the *PTGS2* gene. ChIP was performed with the H14 antibody specific to the Ser-5 phosphorylated CTD of pol-II, and binding was tested between the TATA and E8 sites (mean ± SE, n = 4). Results are presented as in Fig. 4. A, Ser-5 CTD phosphorylation was significantly higher in the TATA and E1 regions than downstream in fresh (0 h) and vehicle-treated (4 h and 24 h) tissues and decreased markedly between 0 h and 4 h ($F_2 = 25.92$; $P < 0.0015$,). B, In dexamethasone-treated tissues (Dex), Ser-5 CTD phosphorylation remained significantly higher at the TATA and E1 regions than further downstream ($F_7 = 6.77$; $P < 0.03$). Comparisons between vehicle and dexamethasone-treated tissues revealed a significant decrease of Ser-5 phosphorylation in the presence of the steroid at 4 h, but not at 24 h ($t_{31} = 2.39$; $P < 0.01$, *t* test with data paired according to amplicons and experiments). Data were transformed for statistical analysis.

24 h, the glucocorticoid had no effect on CTD-Ser-5 phosphorylation compared with the vehicle-treated control.

CTD-Ser-2-phosphorylated (elongating) pol-II distribution at the *PTGS2* gene was measured by ChIP with the phosphorylated-Ser-2-specific antibody H5. CTD Ser-2 phosphorylation exhibited a relatively even distribution along the gene with no consistent indication of higher levels in the TATA and exon 1 regions (Fig 6A). Even distribution of phosphorylated-Ser-2-pol-II was also apparent in dexamethasone-treated tissues (Fig 6B). This is different from the distribution of total pol-II and initiator pol-II, suggesting that a proportion of pol-II recruited to the promoter and/or paused in early transcription is not phosphorylated at Ser-2 and may not be elongation competent. For the same reason, pol-II escape from the paused state on the *PTGS2* gene may be associated with Ser-2 phosphorylation (31). Ser-2 phosphorylation decreased between 0 h and 24 h ($F_2 = 15.39$; $P < 0.001$) in vehicle-treated tissue (Fig 6A), but remained unchanged between 4 h and 24 h in the presence of dexamethasone (Fig 6B). Dexamethasone treatment, however, decreased significantly the level of pol-II CTD Ser-2 phosphorylation along the gene at 4 h of incubation, compared with vehicle ($t_{31} = 2.39$; $P < 0.01$, paired *t* test). The glucocorticoid had no effect on Ser-2 phosphorylation at 24 h.

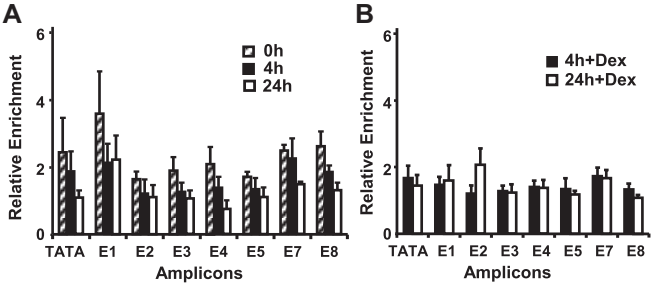

FIG. 6. Serine-2 (elongating) phosphorylation of pol-II at the *PTGS2* gene. ChIP was preformed with the H5 antibody specific to the Ser-2-phosphorylated CTD of pol-II (mean ± SE, n = 4). Results are presented as in Fig. 4. A, Ser-2 phosphorylation was distributed between the TATA and E8 regions, and Ser-2 phosphorylation decreased significantly between 0 h and 24 h of incubation ($F_2 = 15.39$; $P < 0.001$). B, In dexamethasone-treated tissues (Dex), Ser-2 CTD phosphorylation remained even between the TATA and E8 sites. Comparisons between vehicle and dexamethasone-treated tissues revealed a significant decrease of Ser-2 phosphorylation in the presence of the steroid at 4 h, but not at 24 h ($t_{31} = 2.39$; $P < 0.01$, data paired according to amplicons and experiments). Data were transformed for statistical analysis.

Histone acetylation and methylation

We have demonstrated previously that histones H3 and H4 are acetylated at the *PTGS2* promoter in amnion tissue (25). Maximal acetylation of both histones within the 2579-bp upstream promoter region was found at amplicons R3 and R2. Glucocorticoids have been proposed to inhibit gene expression by suppressing histone acetylation at glucocorticoid-sensitive genes (34); therefore we determined whether dexamethasone influenced histone acetylation at R3 and R2 in our amnion incubation system.

H3 and H4 acetylation at the *PTGS2* promoter decreased by approximately 40–50% at 4 h of incubation and remained at this level at 24 h (acH3: $F_2 = 3.75$; $P < 0.05$ and acH4: $F_2 = 3.88$; $P < 0.04$). Dexamethasone had no effect on the H3 or H4 acetylation levels (Fig. 7, panels A and B, respectively).

Methylation of histone 3 at serine 4 (H3K4) in nearby nucleosomes is a marker of transcriptionally active genes (35). We examined H3K4 methylation at the *PTGS2* gene by ChIP with an antibody specific to H3K4me2. Eight PCR amplicons were distributed along the 2579-bp upstream promoter sequence including the TATA-box site, as described previously (25), and a further seven amplicons were located along exons 1–8 as described in Fig. 5B and Supplemental Table 1. We have found a histone methylation signal at the *PTGS2* gene with high levels of H3K4 methylation in the approximately 1000-bp upstream promoter region, around the transcriptional start site (TATA) and at E1. The maximum signal was at sites R3 and R2 (Fig. 8A). H3K4me2 at these sites did not change during incubation in the presence of vehicle or dexamethasone (data not shown).

Discussion

Freshly isolated cells and tissues placed in culture are exposed to a nutritional and hormonal environment that is

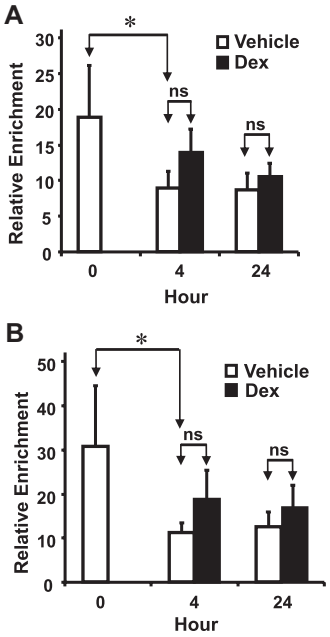

FIG. 7. Acetylation of histone 3 and histone 4 at the *PTGS2* gene. ChIP was performed using antibodies specific to acetylated histone 3 (A) and acetylated histone 4 (B), and binding was tested to amplicon sequences R3 and R2. Relative enrichment was calculated as recovery of input relative to R8. The figures show binding to R3 only, but results for R3 and R2 were combined for statistical analysis. Both histone 3 and histone 4 acetylation decreased significantly between 0 h and 4 h of incubation, and dexamethasone (Dex) had no significant effect on acetylation levels (acH3: $F_2 = 4.43$; $P < 0.02$; and acH4: $F_2 = 3.71$; $P < 0.04$; both marked by asterisk (*); ns, not significant; $P > 0.05$). Statistical analysis was performed with transformed data.

markedly different from the *in vivo* conditions. Survival *in vitro* requires adaptive changes induced by the artificial conditions resulting in model systems that may retain some, but not all, of the original attributes of the cells and tissues. We reasoned that monitoring the adaptive changes during this transitional phase may reveal regulatory features that are important for tissue function *in vivo* but are lost or diminished during adaptation. In the present investigation we used this approach to characterize *PTGS2* gene expression in human amnion from term pregnancies and to clarify the role of glucocorticoids in *PTGS2* gene regulation. These issues have been studied in model systems derived from amnion, but the findings were often conflicting and their relevance to *in vivo* regulation remained unclear (10, 13–15, 17, 18, 20). The fundamental importance of PGs in human parturition warranted further work toward clarification.

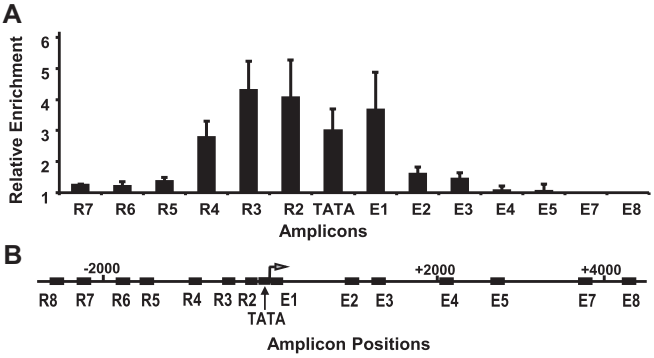

FIG. 8. A, Histone 3-Lysine 4 methylation (H3K4me2) at the *PTGS2* gene. ChIP was performed with an antibody specific to H3K4me2, and relative enrichment was calculated as recovery of input relative to R8 (n = 3 independent experiments). B, Schematic representation of amplicon locations along the *PTGS2* promoter (R8–R2), TATA site and exons 1–8 (E1–E8) used in the ChIP experiments. The exact locations on the gene sequence are listed in Supplemental Table 1. The transcriptional start site is flagged. H3K4 methylation was detected in the region spanning exon 1 and approximately 1 kb upstream of the transcriptional start site.

Amnion tissue was obtained within 30 min after elective cesarean deliveries and incubated in a chemically defined, serum-free nutrient mixture for 24 h as detailed in *Materials and Methods*. Cell viability was not impaired in the explants during the incubation (21); however, a series of changes occurred affecting *PTGS2* gene activity and mRNA abundance. First, *PTGS2* gene activity dropped by approximately 85% during the initial 4-h period without a concomitant drop in the steady-state PTGS2 mRNA level. Subsequently, gene activity rebounded spontaneously and partially (to 25% of the 0 h value), and the rebound was accompanied by a several fold increase in mRNA abundance. These observations corroborate our previous findings (21) and suggest that 1) gene transcription switched to an autonomous mode after 4 h of incubation, and 2) the mature PTGS2 mRNA has stabilized *in vitro*, because the lower level of autonomous gene activity was sufficient to elicit an increase of steady-state mRNA abundance in excess of the 0 h value (representing fresh tissue). We infer that PTGS2 mRNA turnover is faster in the amnion *in vivo* than *in vitro* and that both gene transcription and mRNA breakdown rates are positively regulated in the *in vivo* environment. ChIP data on pol-II distribution revealed accumulation of the enzyme in the region comprising the TATA site and exon 1, which is indicative of postinitiation pausing and possible regulation of pol-II entry into the transcriptional elongation phase. Genome-wide screens showed this type of regulation being relatively widespread (32); however, data involving this mechanism in PTGS2 expression control is scant [*e.g.* reported in HT-29 colon cancer cells (36)], and this is the first time it has been shown in gestational tissues. Pol-II recruitment diminished by 4 h of incubation and remained low afterward, in agreement with the fall in transcriptional activity. Postinitiation pausing, Ser-5 and Ser-2 phosphorylation of pol-II also decreased, and histone acetylation at the promoter region was reduced. Interestingly, the spontaneous transcriptional rebound between 4 h and 24 h was not associated with the reversal of any of these changes, which suggests that the transcriptional apparatus

controlling the *PTGS2* gene has reorganized under the *in vitro* conditions and maintained gene activity at a lower level. It is therefore uncertain whether *PTGS2* transcription after 4 h in culture represents a physiological process or is an adaptive response triggered by the artificial environment.

Our results showed that glucocorticoid (dexamethasone) caused a concentration-dependent decrease of PTGS2 mRNA levels in amnion tissue. We have shown previously that dexamethasone and cortisol inhibited PTGS2 activity, protein levels, and *de novo* enzyme synthesis in amnion explants (14), which was in agreement with observations reported by others (13, 15, 16, 37). The data presented here support strongly the assumption that down-regulation of PTGS2 mRNA abundance and gene activity is responsible, at least in part, for these effects. Thus dexamethasone inhibited *PTGS2* gene transcription, significantly augmenting the spontaneous transcriptional drop at 4 h and preventing the spontaneous rebound at 24 h. The IC_{50} values were in the nanomolar range, which is compatible with a GR-mediated process (38). Accordingly, GRα was bound to the *PTGS2* promoter in fresh tissue. The promoter region in which receptor binding occurred, however, does not contain sequence elements known to bind GRα (glucocorticoid response element) (39, 40), which suggests that binding to DNA was indirect, a characteristic feature of the transrepression of proinflammatory genes by glucocorticoids (22). Glucocorticoid transrepression involves the binding of the GRα-glucocorticoid complex to nuclear factor-κB (NFκB) or activator protein 1 transcription factors associated with proinflammatory gene promoters, thereby blocking the up-regulation of gene activity by these factors (22). NFκB and activator protein 1 have been shown to induce *PTGS2* gene expression in amnion cells, and NFκB binding has been detected to a cognate-binding site by ChIP in the region where we have detected GRα binding (25, 41). We have reported in a previous study, however, that the bound NFκB is not functional *in vivo* (25), which can be explained now by the transrepressive action of GRα.

GRα binding to the promoter diminished by 4 h of incubation but was maintained in the presence of dexamethasone, as expected. Surprisingly, the steroid did not maintain GRα binding at 24 h, although *PTGS2* gene activity remained suppressed, and GRα expression persisted in the tissue explants. This suggests that the GRα-glucocorticoid complex was unable to bind to the molecular apparatus driving the transcriptional rebound between 4 h and 24 h. Pol-II binding studies revealed important details of the mechanism underlying the glucocorticoid repression of *PTGS2* gene activity. Thus, dexamethasone did not alter the amount of pol-II bound to the *PTGS2* gene indicating that the steroid did not affect the recruitment of the enzyme to the promoter. Ser-5 and Ser-2 phosphorylation of pol-II, however, had decreased significantly in dexamethasone-treated tissues at 4 h of incubation, when GRα binding to the promoter was stimulated by glucocorticoid, but not at 24 h, when GRα binding in response to dexamethasone was lost. This suggests that the promoter-bound GRα-glucocorticoid complex interfered with the initiation and elongation functions of pol-II. Glucocorticoids have been reported to repress transcription in other systems by a similar mechanism (42), but in the amnion, these interactions were disrupted rapidly *in vitro* and became undetectable within 24 h. Histone acetylation and methylation (H3K4me2) were unaffected by the steroid at either time point; thus the mechanism by which dexamethasone suppressed transcription at 24 h remains unclear. Notably, our results do not exclude the possibility that dexamethasone facilitated PTGS2 mRNA breakdown because the steroid appeared to down-regulate more effectively the steady-state level of mRNA than the activity of the gene as suggested by the concentration-dependence studies (Fig. 2, A and B). Further work is needed to explore this possibility.

In conclusion, we have presented evidence that PTGS2 mRNA expression in the human amnion is regulated both at the gene transcription and mRNA breakdown levels. We have identified postinitiation pausing of pol-II as a possible regulatory step. *PTGS2* gene transcription and mRNA breakdown rates decrease rapidly during explant incubation and lead to lower mRNA turnover rate and increasing mRNA steady-state levels compared with the *in vivo* situation. The diminished rate of transcription is underpinned by decreasing pol-II recruitment, reduced transcription initiation and elongation, and decreased histone acetylation at the PTGS2 promoter. After 4 h of incubation, transcriptional activity rebounds without the reversal of any of these changes, suggesting that an altered transcriptional apparatus was formed, possibly not modeling the *in vivo* conditions. We have also presented evidence that glucocorticoids inhibit *PTGS2* gene expression in fresh amnion by transrepressing gene activity via the promoter-bound glucocorticoid-GRα complex. The mechanism involves interference with transcription initiation and elongation, but not with pol-II recruitment. Our results do not support the view that glucocorticoids up-regulate PTGS2 expression in amnion tissue as observed in amnion cell cultures (10, 12, 19); however, they are consistent with the clinical observation that glucocorticoids do not induce birth in women. We propose that the principal action of glucocorticoids in amnion is to inhibit PTGS2 expression and PG production similarly to placenta as reported by Siler-Khodr *et al.* (43). Moreover,

endogenous cortisol may chronically inhibit PG synthesis during pregnancy, similar to the chronic antiinflammatory action of glucocorticoids described by Rosen *et al.* (44) in cytotrophoblasts. Establishment of glucocorticoid resistance (45) by physiological or pathological stimuli may contribute to the up-regulation of PTGS2 expression and intrauterine PG production and may promote the initiation of labor.

Acknowledgments

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