Unique Suppression of Prostaglandin H Synthase-2 Expression by Inhibition of Histone Deacetylation, Specifically in Human Amnion but Not Adjacent Choriodecidua

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The key molecular regulatory mechanisms that govern and coordinate the molecular alterations that underpin the process of human labor remain incompletely understood although enhanced intrauterine prostaglandin production is known to be requisite. Studies from cancer tissues have indicated that at least one key enzyme of prostaglandin biosynthesis can have its activity severely reduced by increased histone deacetylation and enhanced DNA methylation status. We have advanced the hypothesis that similar regulation may occur in intrauterine tissues during pregnancy to prevent inadvertent activation of this powerful initiating signal by dampening responses to premature activation by agents such as cytokines. Our studies have shown that responsiveness of amnion, a key intrauterine tissue, to interleukin-1 β is abrogated by inhibition of histone deacetylation, whereas PGDH amounts were increased basally. The findings do integrate well with others concerning progesterone (inhibitory) actions such that a decrease in the level of histone acetylation in human gestational tissues near term might herald a coordinated series of events that all result in a positive drive for parturition. Hence, a new level of regulatory action and potential therapeutic targets for pathologies such as preterm labor can flow from these findings.

INTRODUCTION

The key molecular regulatory mechanisms that govern and coordinate the molecular alterations that underpin the process of human labor remain incompletely understood (Challis *et al.*, 2000; Smith *et al.*, 2002), although enhanced intrauterine prostaglandin production is known to be requisite (Challis *et al.*, 2002; Keelan *et al.*, 2003). There is evidence that the mechanisms of labor and of inflammatory reactions share many elements in common and in specific instances may be overlapping (Mitchell *et al.*, 1983; Bowen *et al.*, 2002). The most discretely recognizable form of preterm labor is that associated with an ascending intrauterine infection and is characterized by enhanced local production of cytokines and raised amniotic fluid levels of such (Romero *et al.*, 1994, 2003). Moreover increased intrauterine cytokine concentrations have been found in association with labor at term (Laham *et al.*, 1996; Keelan *et al.*, 1999). It has been argued also that the withdrawal of inhibitory influences may lead to parturition (Saeed *et al.*, 1982; Lynch-Salamon *et al.*, 1992; Simpson *et al.*, 1998; Blumenstein *et al.*, 2002; Mesiano *et al.*,

2002; Dong *et al.*, 2005) with or without new stimulatory activities.

Recently, it has been shown that the activity of a key prostaglandin (PG) biosynthetic enzyme (prostaglandin H synthase-2; PGHS-2) is suppressed by enhanced DNA methylation and reduced histone acetylation in specific cancer tissues (Toyota *et al.*, 2000; Song *et al.*, 2001; Suzuki *et al.*, 2002). Furthermore, in gastric epithelial cells the regulatory action was most evident when a test stimulatory agent (*Helicobacter pylori*) was added (Akhtar *et al.*, 2001). Moreover, a recent study (Condon *et al.*, 2003) demonstrated that in mice inhibition of histone deacetylation could alter one important inhibitory action viz. enhance progesterone receptor function and possibly through this lengthened gestation.

We hypothesize that in human pregnancy key coordination factors in the maintenance of pregnancy and the onset of labor may be the histone acetylation (and DNA methylation) status of critical prostaglandin biosynthetic genes. To test this hypothesis we have determined the effects of altering the status of both on basal and inflammatory-related stimulation of prostaglandin production and amounts of key biosynthetic enzymes by amnion and choriodecidua, which are critical intrauterine tissue sites of prostaglandin biosynthesis.

MATERIALS AND METHODS

Reagents

5-aza-2' deoxycytidine (ADC), trichostatin A (TSA), and lipopolysaccharide (LPS) were purchased from Sigma Chemical Company (St. Louis, MO). Human recombinant IL-1 β was a generous gift from the Immunex (Seattle, WA). Media were from Irvine Scientific (Santa Ana, CA) and the fetal calf serum

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Abbreviations used: ADC, 5-aza-2' deoxycytidine; TSA, trichostatin A; cPLA2, cytosolic phospholipase A2; PGDH, 15-hydroxyprostaglandin dehydrogenase.

Figure 1. The effects of altering DNA methylation and histone acetylation status on PGE₂ production by human amnion explants. Alterations in DNA methylation and histone deacetylation resulted in a reduction of IL-1 β -induced PGE₂ production by human amnion explants. Basal PGE₂ production was not altered by either treatment (□). In all treatments, there was a statistically significant increase in PGE₂ production upon treatment with IL-1 β (13-fold in controls, ■). IL-1 β treatment resulted in fivefold stimulation with ADC treatment, which is a statistically significant reduction from non-ADC-treated control. PGE₂ production increased only 0.8-fold when explants were stimulated with IL-1 β after ADC and TSA treatment, which is almost a complete abrogation of the response and again statistically significantly reduced when compared with the 5 μ M ADC treatment or control. Data are presented as the production of PGE₂ (pg/mg wet weight/24 h); * $p < 0.05$, $n = 5$.

was from Invitrogen (Grand Island, NY). Tritiated PGE₂ was purchased from Amersham-Pharmacia Biotech (Aylesbury, United Kingdom). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; PGHS-1 and 2, and to cytosolic phospholipase A₂ [cPLA₂], Santa Cruz). An antibody to 15-hydroxyprostaglandin dehydrogenase (PGDH) was purchased from Cayman Chemicals (Ann Arbor, MI).

The Auckland Ethics Committee approved all procedures involving human placentas. Placentas were obtained from women undergoing elective Caesarean section at term. Amnion and choriodecidual explants were prepared by routine methods (Simpson *et al.*, 1998). Explants were treated with various concentrations and a combination of 5-aza-2'-deoxycytidine (ADC) an inhibitor of DNA methylation or a much lower concentration of ADC plus TSA, an inhibitor of histone deacetylation, using established effective concentrations (Condon *et al.*, 2003). Specifically, explants were treated with ADC (5 μ M or 200 nM) for 48 h and kept in 5% CO₂ in air at 37°C, the media were replaced daily. After 48 h, amnion explants were treated with IL-1 β (1 ng/ml) and choriodecidual explants with LPS (5 μ g/ml; Sato *et al.*, 2003). Additionally, the explants treated with 200 nM ADC had TSA (300 nM) added also (Cameron *et al.*, 1999). After further 24-h incubation, the media were removed and the wet weight of the tissue in each well determined so that production rates could be normalized.

Immunoassays

PGE₂ was measured by a sensitive and specific radioimmunoassay that we have developed and validated in our laboratory. The assay has a sensitivity of ~7 pg/ml (Sato *et al.*, 2003).

Western Blot Analysis

Total amnion and choriodecidual explant proteins were prepared by published methods and Western blot analysis was conducted (Blumenstein *et al.*, 2002). Densitometric analysis was performed using the Labworks program (UVP, Upland, CA).

Presentation of Data

Production rates of prostaglandins were calculated as pg/mg wet tissue weight/24 h ($n = 3$ or 5, mean $\pm$ SEM). PGHS-1, PGHS-2, cPLA₂, and PGDH were measured as optical density (OD) units and expressed as a ratio over β -actin to account for loading differences ($n = 3$). Statistical significance was determined by ANOVA; $p < 0.05$ was considered to be significant.

RESULTS

Treatment of amnion explants with IL-1 β resulted in a 13-fold stimulation of PGE₂ production which was substan-

Figure 2. The effects of altering DNA methylation and histone acetylation status on PGHS-1 and PGHS-2 expression by human amnion explants. Altering the DNA methylation or histone deacetylation status in human amnion explants effects the expression of PGHS-1 and PGHS-2. Basal expression (□) of PGHS-1 was significantly reduced when the DNA methylation status was altered. This was also observed when the explants were stimulated with IL-1 β (■). Explants were cultured for 24 h after the addition of IL-1 β . Alterations of the histone deacetylation status did not effect basal expression of PGHS-1; however, there was a significant reduction when the human amnion explants were stimulated with IL-1 β . There was no observed effect on PGHS-2 expression upon altering the DNA methylation and histone deacetylation status of human amnion explants. Stimulation with IL-1 β resulted in a significant increase in PGHS-2 expression. Alteration of DNA methylation had no effect on IL-1 β -induced PGHS-2 expression; however, altering the histone deacetylation resulted in a significant reduction of PGHS-2 expression. Data are presented as the expression of PGHS-1 or PGHS-2 divided by the expression of β -actin to account for differences in loading. * $p < 0.05$ compared with untreated control # $p < 0.05$ compared with IL-1 β -treated control, $n = 3$.

tially reduced by inhibition of DNA methylation via cotreatment with ADC and massively abrogated by the inhibition of histone deacetylation through a combination of ADC and TSA (Figure 1); in further experiments, TSA alone almost completely abrogated the stimulatory actions of IL-1 β to the same extent as TSA in combination with ADC (unpublished data).

We next sought to determine the level(s) in the prostaglandin biosynthetic pathways at which these effects were manifested. Treatment with IL-1 β had no significant effect on the amounts PGHS-1. Neither inhibition of DNA methylation nor histone deacetylation altered PGHS-1 amounts

Figure 3. The effects of altering DNA methylation and histone acetylation status on cPLA₂ and PGDH expression by human amnion explants. Altering the DNA methylation or histone deacetylation status in human amnion explants had no effect on basal (□) or in the presence of IL-1 β (■) cPLA₂ expression in amnion explants. Explants were cultured for 24 h after the addition of IL-1 β . Neither altering the DNA methylation or histone deacetylation status of human amnion explants effected cPLA₂ expression. Stimulation with IL-1 β resulted in a statistically significant increase in PGDH expression by human amnion explants. Basally, altering the DNA methylation and histone deacetylation status also resulted in a statistically significant decrease in PGDH expression. Histone deacetylation resulted in a further reduction in PGDH expression when there was a stimulation with IL-1 β . * $p < 0.05$ compared with untreated control; # $p < 0.05$ compared with IL-1 β -treated control, $n = 3$.

(Figure 2). The results with PGHS-2 were revealing. Inhibition of DNA methylation was without effect on the stimulatory actions of IL-1 β but inhibition of histone deacetylation completely abolished the stimulatory action of IL-1 β (Figure 2).

We next evaluated the effect of these treatments on cPLA₂. The results were unremarkable (Figure 3), although it had been anticipated that IL-1 β would increase cPLA₂ expression. Finally, PGDH expression was evaluated. Treatment with IL-1 β caused a reduction in the amount of PGDH. Both cotreatments abrogated that inhibitory effect of IL-1 β primarily by reducing basal amounts of PGDH. (Figure 3).

We conducted essentially the same series of studies as had been performed with amnion using choriodecidual explants to eval-

Figure 4. The effects of altering DNA methylation and histone acetylation status on PGE₂ production by human choriodecidual explants. Alterations in DNA methylation and histone deacetylation effected basal PGE₂ production by human choriodecidual explants. Basal production of PGE₂ (□) statistically increased when the DNA methylation and histone deacetylation status of human choriodecidual explants were altered. In all treatments, stimulation with LPS (■) resulted in a significant increase in PGE₂ production; however, altering the DNA methylation or histone deacetylation did not affect the stimulated levels of PGE₂ produced. Data are presented as the production of PGE₂ as a percentage of control values. * $p < 0.05$, $n = 3$.

uate whether effects observed with amnion were specific to that tissue or reflective of an intrauterine environment.

Treatment with LPS resulted in an approximately seven-fold increase in PGE₂ production. (Figure 4). Both inhibition of DNA methylation and of histone deacetylation caused a significant doubling of the basal rate of PGE₂ production without altering the level of production in response to LPS. Thus the degree of stimulation of PGE₂ production was reduced by half by these two cotreatments. This is a very different pattern of responses from that of amnion. Both inhibition of DNA methylation and histone deacetylation reduced PGHS-1 (Figure 5) amounts but had no effect on the slight inhibitory action of LPS although the latter treatment did exacerbate this effect to some extent

Despite multiple attempts and refinements we were unable to detect a signal for cPLA₂ in the choriodecidual explants. Surprisingly, there does not appear to be many studies to which we can refer to confirm or refute this finding. In direct contrast to amnion, the choriodecidual responded to LPS treatment with significantly greater amounts of PGDH. (Figure 6). This effect was washed out by inhibition of DNA methylation and indeed reversed by inhibition of histone deacetylation, although the major effect was a substantial increase in basal PGDH amounts.

DISCUSSION

These results suggest that if the reduced human uterine tissue levels of acetylated histone H3 noted by Condon *et al.* (2003) were reflected in other key sites that this could constitute a cardinal coordinating regulatory signal for the onset of labor. Massive attenuation of potential stimulatory drivers (PGs) to the labor process affected via histone acetylation status may have been revealed by the use of TSA. Hence enhanced responsiveness of key uterine contractile prostaglandins and diminished functionality of uterine quiescence systems (i.e., progesterone; Mesiano *et al.*, 2002; Dong *et al.*, 2005) are both coordinated via reduced histone acetylation levels or the integrated response of corepressor and coactivator complexes with an array of signal transduction pathways (Torchia *et al.*, 1998). Further detailed investigation will revolve around the roles of specific histone deacetylases (Thaigalingam *et al.*, 2003). Moreover, because significant

Figure 5. The effects of altering DNA methylation and histone acetylation status on PGHS-1 and PGHS-2 expression by human amnion explants. Alterations of DNA methylation and histone deacetylation had effects on PGHS-1 expression upon stimulation with LPS (■), but not basally (□). Explants were cultured for 24 h after the addition of LPS. Altering the DNA methylation or histone deacetylation status of human chorionic explants had no effect on the basal expression of PGHS-1. LPS stimulation of human chorionic explants had no effect on PGHS-1 expression; however, treatment with ADC or ADC+TSA resulted in a significant decrease in PGHS-1 expression. No treatment examined had any significant effects on PGHS-2 expression. # $p < 0.05$ compared with LPS treated control, $n = 3$.

effects were noted with ADC, we will be determining the roles of DNA methylation transferases with a focus on Dnmt3a and Dnmt3b (Oka *et al.*, 2005). A further refinement in linking these overall events has come from the recent finding that enhanced prostaglandin production may effect functional progesterone withdrawal via differential actions on receptor isoforms (Madsen *et al.*, 2004). Cytokine stimulation of PGHS-2 mRNA in amnion occurs via C/EPB β (Potter *et al.*, 2000) activation and this is suppressed by inhibitors of histone deacetylation (Desilets *et al.*, 2000; Xu *et al.*, 2003). Hence histone acetylation of key genes may maintain the reduced intrauterine PG environment required for maintenance of pregnancy (Maathuis and Kelly, 1978) particularly in the face of external stimuli such as cytokines. This would be affected via coordinated and reciprocal actions on biosynthesis (PGHS-2) and metabolism (PGDH) and may be reinforced by factors such as the potential antagonistic action of PGDH on PGHS-2 activity (Yan *et al.*, 2004).

Figure 6. The effects of altering DNA methylation and histone acetylation status on cPLA2 and PGDH expression by human chorionic explants. Alterations in histone deacetylation status of human chorionic explants effects the basal (□) expression of PGDH. cPLA2 expression was also examined, but there were no detectable levels in human chorionic explants. PGDH expression was significantly increased when the histone deacetylation status was altered. On stimulation with LPS (■), there was a statistically significant increase in PGDH expression, treatment with ADC or ADC+TSA had no effect on the LPS-induced expression levels. Explants were cultured for 24 h after the addition of LPS. * $p < 0.05$ compared with untreated control, $n = 3$.

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