Induction of progesterone receptor A form attenuates the induction of cytosolic phospholipase A_{2α} expression by cortisol in human amnion fibroblasts

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Abstract

Cytosolic phospholipase A_{2α} (cPLA_{2α}, now known as PLA2G4A) is the enzyme catalyzing the formation of the rate-limiting substrate, arachidonic acid, for prostaglandin (PG) synthesis. The increasing expression of PLA2G4A toward term gestation in human amnion fibroblasts is believed to be the crucial event in parturition. Human amnion fibroblasts produce cortisol, progesterone and express glucocorticoid receptor (GR), progesterone receptor A (PGRA) form at term. The roles of progesterone and PGRA in the induction of PLA2G4A by cortisol via GR in the amnion fibroblasts remain largely unknown. Using cultured human term amnion fibroblasts, we found that cortisol induced the expression of PGRA, which was attenuated by inhibiting PG synthesis with indomethacin. Knockdown of PGRA expression or inhibition of endogenous progesterone production with trilostane significantly enhanced the induction of PLA2G4A by cortisol, whereas overexpression of PGRA attenuated the induction of PLA2G4A by cortisol. Although exogenous progesterone did not alter PLA2G4A expression under basal conditions, it attenuated cortisol-induced PLA2G4A expression at concentrations about tenfold higher, which might be achieved by competition with cortisol for GR. In conclusion, PGRA in the presence of endogenous progesterone is a transdominant repressor of the induction of PLA2G4A by cortisol. High level of progesterone may compete with cortisol for GR, thus further inhibiting the induction of PLA2G4A by cortisol. Moreover, increased PG synthesis by cortisol may feed back on the expression of PGRA leading to attenuation of cortisol-induced PLA2G4A expression. The above findings may be pertinent to the inconsistent effects of glucocorticoids on parturition in humans.

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Introduction

In many animal species, as represented by sheep, parturition is initiated by the fetus, and it depends on an intact and maturing fetal hypothalamus/pituitary/adrenal axis producing increasing amount of glucocorticoids and culminating a glucocorticoid surge before parturition (Challis *et al.* 1977, Jenkin & Young 2004). This glucocorticoid surge induces the cytochrome P450 17 α -hydroxylase/C17–20 lyase in the placenta, which converts progesterone into estrogen, thus leading to progesterone withdrawal and increased estrogen synthesis before parturition (Challis *et al.* 1977, Jenkin & Young 2004). In addition, glucocorticoids also act to increase the production of prostaglandins (PGs) in the intrauterine tissues. Consequently, PGs stimulate myometrial contraction, cervical ripening, rupture of fetal membranes (Challis *et al.* 2002), and luteolysis in animals that progesterone is produced by the corpus luteum during pregnancy (Buckle & Nathanielsz 1973, Shaikh *et al.* 1977). Thus, glucocorticoids derived from

the maturing fetal adrenals are believed to play a triggering role in parturition in these animal models. However, in higher primates including humans, the role of fetus in initiating parturition is rather intriguing. In contrast to the sheep model, dexamethasone administered to pregnant rhesus monkeys near term fails to induce premature parturition (Challis *et al.* 1974). Moreover, the efficacy of intra-amniotic administration of glucocorticoids for induction of labor is less consistent in humans (Mati *et al.* 1973, Katz *et al.* 1979). In spite of these findings, glucocorticoids are proved to stimulate the production of tocolytic hormones such as PG and CRH in human intrauterine tissues *in vitro* (Potestio *et al.* 1988, Robinson *et al.* 1988, Mclean *et al.* 1995, Economopoulos *et al.* 1996, Sun *et al.* 2003). Causes for the inconsistent effects of glucocorticoids in human parturition are not fully understood.

Owing to a lack of the glucocorticoid target enzymes, P450 17 α -hydroxylase/C17–20 lyase, in human placenta, maternal progesterone level remains elevated during labor and delivery (Tulchinsky *et al.* 1972,

Figure 1 (A and B) Treatment of human amnion fibroblasts with cortisol for 24 h increased *PGRA* mRNA ($n=4$) and protein ($n=5$) levels in a concentration-dependent manner, which were attenuated by RU486. (C and D) Treatment of human amnion fibroblasts with indomethacin (Indo, 10 μ M) inhibited PGE₂ production ($n=4$) and the induction of *PGRA* protein expression by cortisol (F, 1 μ M) for 24 h ($n=4$). (E) Cortisol (1 μ M) treatment of human amnion fibroblasts for 24 h induced *PGRA* expression while attenuated *GR* expression. Top panels of B and D are the representative immunoblots. * $P < 0.05$ versus vehicle control (ctr) without cortisol and RU486; # $P < 0.05$ versus cortisol (1 μ M) without RU486 or without indomethacin.

Walsh *et al.* 1984). Thus, unlike in most of other species that progesterone withdrawal is achieved by a rapid fall in progesterone level prior to the onset of labor, the progesterone withdrawal in humans has been suggested to be achieved functionally via the expression of progesterone receptor A (PGRA) form under the influence of PGs in the myometrium (Allport *et al.* 2001, Madsen *et al.* 2004). PGRA is a N-terminal truncated form of the classical progesterone receptor B (PGRB) form, lacking 164 amino acids at the N-terminus compared to the full-length PGRB (Graham & Clarke 2002). While the two PGR forms have similar DNA- and ligand-binding affinities and are able to form homo- and/or heterodimers, the truncated PGRA has poor transcriptional activity (Vegeto *et al.* 1993). PGRA has been reported to antagonize the classical transcriptional activities of PGRB by forming heterodimers with PGRB, which diminishes myometrial progesterone responsiveness (Haluska *et al.* 2002, Merlino *et al.* 2007). However, in addition to PGRB, PGRA has been reported to be a transdominant inhibitor of most of the other steroid receptors including glucocorticoid receptor (GR), estrogen receptor (ER), androgen receptor (AR), and

mineralocorticoid receptor (MR; Vegeto *et al.* 1993, Wen *et al.* 1994).

We have demonstrated that glucocorticoids stimulate PG production by inducing the expression of cytosolic phospholipase A_{2 α} (cPLA_{2 α} , now known as PLA2G4A; Sun *et al.* 2003) as well as cyclooxygenase-2 (COX-2, now known as PTGS2; Potestio *et al.* 1988, Economopoulos *et al.* 1996, Sun *et al.* 2003) via GR in human amnion fibroblasts. Recently, we demonstrated that term human amnion fibroblasts express mainly PGRA but not PGRB, and upon binding by endogenous progesterone, PGRA counteracts the induction of PTGS2 by cortisol via transdominant repression of GR function (Guo *et al.* 2009). Whether this phenomenon holds true in the regulation of PLA2G4A expression in human amnion fibroblasts is unknown. As have been reported that the functional progesterone withdrawal is facilitated by PGs via increasing the expression ratio of PGRA/PGRB in human myometrium (Madsen *et al.* 2004), thus it is very likely that the expression of PGRA is under the control of glucocorticoids in this cell type since glucocorticoids are potent stimulators of PG synthesis in human amnion fibroblasts. To address the above issues, we investigated the effect of glucocorticoids on the expression of PGRA as well as the role of PGRA in the induction of PLA2G4A expression by glucocorticoids in human amnion fibroblasts.

Figure 2 (A) Representative immunoblots showing the decreased expression of PGRA by *PGR* siRNA transfection in comparison with scrambled siRNA negative control (NC) in human amnion fibroblasts. (B–D) Transfection of human amnion fibroblasts with *PGR* siRNA enhanced the induction of PLA2G4A promoter activity ($n=3$), mRNA ($n=3$), and protein expression ($n=4$) by cortisol (F, 1 μ M) for 24 h. Top panel of D is the representative immunoblots. ** $P < 0.01$ versus respective controls; * $P < 0.05$, ** $P < 0.01$ versus negative control with cortisol.

Figure 3 (A) Representative immunoblots showing the increased expression of PGRA by pBKC-PGRA transfection in comparison with empty vector negative control (NC). (B–D) Transfection of human amnion fibroblasts with pBKC-PGRA attenuated the induction of PLA2G4A promoter activity ($n=3$), mRNA ($n=3$), and protein expression ($n=4$) by cortisol (F, 1 μ M) for 24 h. Top panel of D is the representative immunoblots. ** $P<0.01$ versus respective controls; # $P<0.05$ versus negative control with cortisol.

Results

Induction of PGRA expression by cortisol in human amnion fibroblasts

Treatment of human amnion fibroblasts with cortisol (0.01–1 μ M) for 24 h increased PGRA mRNA and protein levels in a concentration-dependent manner (Fig. 1A and B). RU486 (1 μ M) attenuated the induction of PGRA mRNA and protein by cortisol (Fig. 1A and B), suggesting the involvement of GR in the induction. Inhibition of PG synthesis with indomethacin (10 μ M) dramatically decreased the PGE₂ level in the culture medium despite varied basal PGE₂ levels in the four patients (Fig. 1C) as well as the induction of PGRA by cortisol (1 μ M; Fig. 1D), suggesting the involvement of PGs in the induction of PGRA expression by cortisol. In contrast to the induction of PGRA, the expression of GR was attenuated by cortisol treatment for 24 h (Fig. 1E).

Over- or knockdown PGRA expression on the induction of PLA2G4A expression by cortisol in human amnion fibroblasts

Transfection of siRNA or pBKC-PGRA plasmid caused an average of 41.7% decrease or 60.5% increase in PGRA protein level respectively (Figs 2A and 3A). Attenuation of

PGRA expression significantly enhanced the induction of PLA2G4A promoter activity, mRNA, and protein expression by cortisol (1 μ M; Fig. 2B–D), whereas overexpression of PGRA significantly attenuated the induction of PLA2G4A promoter activity, mRNA, and protein expression by cortisol (1 μ M; Fig. 3B–D). These results suggest that PGRA expressed in human amnion fibroblasts may act as a transdominant repressor of the transcriptional activity of GR in the regulation of PLA2G4A expression by cortisol.

Effect of progesterone on the induction of PLA2G4A expression by cortisol in human amnion fibroblasts

Blocking the endogenous production of progesterone with trilostane (1 μ g/ml) had no effect on the basal PLA2G4A mRNA and protein expression, but enhanced the induction of PLA2G4A promoter activity, mRNA, and protein expression by cortisol (1 μ M; Fig. 4A and B). Treatment of the cells with exogenous progesterone (0.1,

Figure 4 (A) Treatment of human amnion fibroblasts with trilostane (Tril, 1 μ g/ml) enhanced the induction of PLA2G4A promoter activity (open columns, $n=3$) and mRNA (filled columns, $n=5$) expression by cortisol (F, 1 μ M) for 24 h. (B) Treatment of human amnion fibroblasts with trilostane (1 μ g/ml) enhanced the induction of PLA2G4A protein expression by cortisol (1 μ M) for 24 h ($n=3$). Top panel of B is the representative immunoblots. ** $P<0.01$ versus vehicle control (ctr); # $P<0.05$ versus cortisol without trilostane.

Figure 5 Treatment of human amnion fibroblasts with exogenous progesterone (0.1–10 μM) for 24 h on the basal and cortisol (0.1 μM)-induced PLA2G4A promoter activity (A, $n=3$), mRNA (B, $n=4$), and protein expression (C, $n=3$). Top panel of C is the representative immunoblots. * $P<0.05$, ** $P<0.01$ versus vehicle control without progesterone and cortisol; # $P<0.05$, ## $P<0.01$ versus cortisol without progesterone.

1, and 10 μM) failed to affect the basal PLA2G4A promoter activity, mRNA, and protein expression (Fig. 5A–C). However, exogenous progesterone at concentrations tenfold higher than cortisol was able to block the induction of PLA2G4A promoter activity, mRNA, and protein expression by cortisol (Fig. 5A–C).

Discussion

It is believed that the stimulation of PG production by glucocorticoids in the amnion comprises the cascade feed-forward events in parturition (Challis *et al.* 1977). The amnion, particularly amnion fibroblasts, is the major

source of PGs toward the end of gestation and during parturition in humans (Whittle *et al.* 2000, Sun *et al.* 2003). Glucocorticoids have been shown to increase the production of PGs in human amnion fibroblasts *in vitro* (Potestio *et al.* 1988, Economopoulos *et al.* 1996, Whittle *et al.* 2000, Sun *et al.* 2003, Guo *et al.* 2008). However, the role of glucocorticoids in human parturition is not as evident as in other species of animals (Liggins & Howie 1972, Whittle *et al.* 2001). Some studies demonstrated that glucocorticoids were able to induce labor in humans as well as in the sheep (Mati *et al.* 1973, Elliott & Radin 1995), while others failed to demonstrate any labor-inducing effect of glucocorticoids in humans (Katz *et al.* 1979). Causes for the disparity are not very well understood. In marked contrast to most of other animal species in which there is a progesterone level declination prior to parturition, there are concurrent increases in progesterone and cortisol levels toward the end of human pregnancy. Increasing expression of PGRA in the myometrium or declines of PGR coactivators such as steroid receptor coactivators (SRC-1 and SRC-3) and cAMP response element-binding protein have been suggested to account for the functional withdrawal of progesterone at the end of human gestation (Haluska *et al.* 2002, Condon *et al.* 2003, Merlino *et al.* 2007). Our previous study, Guo *et al.* (2009) demonstrated that human amnion fibroblasts expressed only PGRA but not PGRB. Although the transition of PGRB to PGRA in the myometrium has been suggested to be in favor of the functional withdrawal of the quiescent effect progesterone in the myometrium (Madsen *et al.* 2004, Merlino *et al.* 2007), the expression of PGRA in the amnion fibroblasts appears to jeopardize the actions of glucocorticoids in the regulation of PG synthesis. PGRA is capable of inhibiting the transcriptional activity of most of the steroid receptors including GR, MR, ER, and AR as well as PGRB in virtually all the cell types examined (Vegeto *et al.* 1993, Wen *et al.* 1994, Graham & Clarke 2002). In this study, we demonstrated an inhibitory effect of PGRA on the induction of PLA2G4A expression by cortisol, which resembles the effect of PGRA on the induction of PTGS2 expression by cortisol in human amnion fibroblasts (Guo *et al.* 2009). It has been proved that the induction of both PLA2G4A and PTGS2 expression by glucocorticoids is mediated by GR in human amnion fibroblasts (Potestio *et al.* 1988, Sun *et al.* 2003, Guo *et al.* 2008). Thus, we believe that this repressing effect of PGRA on the effects of glucocorticoids is exerted via a transdominant inhibitory interaction between PGRA and GR in human amnion fibroblasts. Of interest, PGRA has been demonstrated to antagonize NFκB expression, the key nuclear factor mediating the induction of PG synthesis by proinflammatory cytokines, in myometrium (Hardy *et al.* 2006), which may provide an alternative way of PGRA attenuating PLA2G4A expression.

A previous study demonstrated that the repressor function of PGRA on GR-mediated effects might not require the direct DNA binding, since the compound ZK98299 interfering with PGRA binding of DNA or introduction of point mutations into the DNA-binding domain of PGRA does not affect PGRA transdominant repressor function (Vegeto *et al.* 1993). Rather, the transdominant repressing effect of PGRA has been suggested to be exerted through competition with other steroid receptors for a common transcription factor or alternatively formation of heterodimers with other steroid receptors, thus blocking their transcriptional activities (Wen *et al.* 1994). These latter mechanisms appear to be true in the case of PGRA and GR interaction in this study, as our previous study has shown that PGRA and GR were found in the same protein complex precipitated by GR antibody in human amnion fibroblasts (Guo *et al.* 2009).

Although the lack of the N-terminal 164 amino acids compromises the transcriptional activity of PGRA, PGRA has normal ligand-binding affinity. It has been reported that the transdominant repressor function of PGRA requires the presence of physiological concentration of progesterone (Vegeto *et al.* 1993). Our findings that blocking the endogenous progesterone production with trilostane enhanced the stimulatory effect of cortisol on PLA2G4A expression support this notion. However, further increase in progesterone concentration by adding exogenous progesterone did not lead to further inhibition of GR-mediated induction of PLA2G4A unless progesterone reached concentrations tenfold higher than cortisol, suggesting that the level of endogenous progesterone is high enough to activate the transdominant effect of PGRA. Since progesterone is known to have an affinity for GR about one-fourth of that of cortisol for GR (Song *et al.* 2001), we believe that the inhibitory effect of exogenous progesterone at high concentrations on cortisol-induced expression of PLA2G4A was achieved via competitive binding to GR. As the concentration of progesterone can rise up to micromolar concentrations in both maternal and fetal circulations toward the end of gestation (Walsh *et al.* 1984), this competitive binding of GR by cortisol and progesterone may have physiological impact on parturition. In addition, this competition may also account, at least in part, for the successful intervention of preterm delivery with progesterone analogs in clinical trials (Meis *et al.* 2003, Moster *et al.* 2008).

Of interest, we demonstrated in this study that the expression of PGRA was increased while the expression of GR was decreased by cortisol treatment for 24 h in human amnion fibroblasts. The induction of PGRA by cortisol was blocked by inhibition of PG synthesis with indomethacin. Thus, this effect was very likely to be secondary to the increased PG synthesis upon cortisol stimulation of the cells, as PGs have been shown to be involved in the increased ratio of PGRA/PGRB in the

myometrium (Madsen *et al.* 2004). This induction of PGRA by cortisol may further compromise the induction of PLA2G4A expression by cortisol.

However, it should also be kept in mind that the amnion fibroblasts express abundant 11 β -hydroxysteroid dehydrogenase type 1 (HSD11B1; Sun *et al.* 1997, Sun & Myatt 2003), which converts biologically inactive cortisone to active cortisol. Since glucocorticoids exert potent induction of HSD11B1 in human amnion fibroblasts (Sun & Myatt 2003), cortisol derived from this enzyme may, on the other hand, allow for less progesterone to impair the induction of PG synthesis by cortisol *in vivo*. Thus, the simultaneous induction of PGRA and HSD11B1 as well as attenuation of GR expression by cortisol in the amnion should be taken into consideration when evaluating the net effect of cortisol and progesterone interactions on progesterone production *in vivo*.

Our results substantiate the conclusion that PGRA in the presence of endogenous progesterone is a transdominant repressor of GR-mediated induction of PLA2G4A expression by cortisol in human amnion fibroblasts. Further increase in progesterone concentration may compete with cortisol for binding with GR, thereby further inhibiting the induction of PLA2G4A expression by cortisol. Moreover, the increased PG synthesis stimulated by cortisol may feedback on the expression of PGRA leading to further attenuation of GR-mediated induction of PLA2G4A by cortisol. Taken all these results together, the inhibitory actions of progesterone and PGRA on GR-mediated induction of PLA2G4A expression by cortisol may partly explain the inconsistent effect of glucocorticoids on parturition in humans.

Materials and Methods

Human amnion fibroblast cell culture

Fetal membranes were collected from patients undergoing elective cesarean section at term under a protocol approved by the ethics committee of the School of Life Sciences, Fudan University. All patients gave informed, written consent. Patients who were treated with steroids or other anti-inflammatory agents or with clinical indication of inflammation were excluded from this study. Amnion was peeled off the chorion and digested twice with 0.125% trypsin (Sigma). The digestion medium was discarded, and the amnion tissue was washed thoroughly with PBS to get rid of residual epithelial cells. The remaining amnion tissue was further digested with 0.1% collagenase (Roche). The fibroblast cells in the digestion medium were purified with discontinuous Percoll (GE Healthcare, Uppsala, Sweden) gradients (5, 20, 40, and 60%) and cultured in the complete medium consisted of DMEM (Gibco), 10% newborn calf serum (NCS; Gibco), and antibiotic–antimycotic (Gibco). The detailed method and identity of cells have been described previously (Sun *et al.* 2003).

Treatment of human amnion fibroblasts and determination of *PGRA* mRNA and protein levels

On the third day of amnion fibroblast culture, the culture medium was changed to NCS-free medium. To examine the effect of cortisol on *PGRA* mRNA and protein expression, the cells were treated with cortisol (Sigma) in the presence or absence of RU486 (Sigma) for 24 h. To explore whether the effect of cortisol on *PGRA* expression is dependent on the production of PGs, the cells were pretreated with indomethacin (10 µM; Calbiochem, La Jolla, CA, USA) for 30 min and then continued with cortisol for another 24 h. The effect of indomethacin on PG production was determined by measuring PGE₂ level in the culture medium with an enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA).

After treatment, total RNA was extracted from the cells using a UNIQ-10 RNA extraction kit (Sangon Biotech, Shanghai, China). mRNA (1.0 µg) was reverse-transcribed to cDNA with oligo (dT)12–18 primer using Moloney murine leukemia virus reverse transcriptase (Promega). The mRNA levels of *PGRA* were determined with quantitative real-time PCR (qRT-PCR) using power SYBR green PCR master mix (Toyobo, Osaka, Japan). The annealing temperature was set at 61 °C. The absolute mRNA levels in each sample were calculated according to a standard curve set up using serial dilutions of known amounts of specific templates against corresponding cycle threshold values. To control sampling errors, qRT-PCR for β -actin was routinely performed on each sample. The primer sequences for amplifying *PGRA* and β -actin are illustrated in Table 1. Although the *PGRA* primers span both *PGRA* and *PGRB*, the PCR product represented *PGRA* rather than *PGRB* since our previous study has proved that human amnion fibroblasts express *PGRA* but not *PGRB* at term (Guo *et al.* 2009).

Following treatment, total protein was extracted from the cells using a protein extraction kit (Active Motif, Carlsbad, CA, USA). The expression of *PGRA* protein was examined following a standard western blotting protocol. Briefly, 50 µg protein of each sample were electrophoresed in 6.0–7.5% SDS-polyacrylamide gel and transferred to the nitrocellulose blot. After blocking, the blot was probed with 1:500 dilution of PGR antibody detecting both *PGRA* (95 kDa) and *PGRB* (105 kDa; sc-539, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or GR antibody (sc-E20, Santa Cruz) overnight. After incubation with appropriate secondary antibody conjugated with HRP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), the enhanced chemiluminescence detection system

(Millipore, Billerica, MA, USA) was used to detect the bands. The same blot was reprobed with β -actin antibody (sc-47778, Santa Cruz) for loading control examination.

Transient transfection of human amnion fibroblasts with pGL3-enhancer plasmid carrying *PLA2G4A* promoter-driven luciferase reporter gene

On the second day of culture, the cells were co-transfected with 0.5 µg/well of pGL3-enhancer plasmid carrying *PLA2G4A* promoter (–505 bp)-driven luciferase reporter gene and 0.05 µg/well of pSV- β -galactosidase plasmid using lipofectamine LTX in Opti-MEM (Invitrogen). In another set of experiments, the cells were co-transfected with the above plasmids and *PGRA* siRNA or vector expression *PGRA* (described below). The above plasmids were extracted from the transformed bacteria using endotoxin-free Nucleobond PC500 EF Kit (Macherey-Nagel, Duren, Germany). The pSV- β -galactosidase plasmid was used for transfection efficiency control. Forty-eight hours after transfection, the cells were treated with cortisol (0.1 µM) in the presence and absence of progesterone (0.1–10 µM), or with cortisol (1 µM) in the presence and absence of trilostane (1 µg/ml; Sterling, Winthrop Research Institute, New York, NY, USA), an inhibitor of endogenous progesterone synthesis (le Roux *et al.* 2002), in serum-free medium for 24 h. The cells were then lysed for subsequent measurements of luciferase activity using Luciferase Assay System (Promega) and β -galactosidase activity using β -galactosidase Enzyme Assay System (Promega).

Determination of the effect of knockdown or overexpression of *PGRA* on the induction of *PLA2G4A* mRNA and protein by cortisol in human amnion fibroblasts

To study the role of *PGRA* in the regulation of *PLA2G4A* mRNA and protein expression by cortisol, predesigned siRNA (10 nM) against *PGR* (s10415; Ambion, Austin, TX, USA) or pBKC-*PGRA* vector expressing *PGRA* (kindly provided by Dr D McDonnell, Duke University Medical Center, Durham, NC, USA) was utilized to transfect human amnion fibroblasts with Lipofectamine LTX (Invitrogen) in Opti-MEM. Randomly scrambled negative control siRNA or empty vector was utilized for controls for *PGRA* knockdown and overexpression studies respectively. Forty-eight hours after transfection, the cells were challenged with cortisol (1 µM) for 24 h in serum-free medium,

Table 1 Primer sequences used for quantitative real-time PCR.

Genes	Primer sequences	GenBank accession no.	Size of PCR products (bp)
<i>PGRA</i>	F: 5'-CTGCAGGTCTACCCGCCCTATC-3' R: 5'-GAATTCATTTGGAACGCCCACTGG-3'	NM_000926	396
<i>PLA2G4A</i>	F: 5'-ATGGCCTTGGTGAGTGATTC-3' R: 5'-TCAGGATCTGCTACAGCTGC-3'	NM_024420	179
β -Actin	F: 5'-GGGAAATCGTCGTGACATTAAG-3' R: 5'-TGTGTTGGCGTACAGGTCTTTG-3'	NM_001101	275

F, forward; R, reverse.

and were collected for total RNA and protein extractions. The efficiency of siRNA and pBKC-PGRA vector transfection was determined by measuring the PGRA protein level. The PGRA and PLA2G4A protein levels were determined with western blotting, and the PLA2G4A mRNA level was determined with qRT-PCR as described above. The PLA2G4A antibody used for western blotting was purchased from Santa Cruz (sc-454), and the primers used for amplifying PLA2G4A with PCR were illustrated in Table 1.

Determination of the effects of endogenous and exogenous progesterone on the induction of PLA2G4A expression by cortisol in human amnion fibroblasts

Our previous study has demonstrated that human amnion fibroblasts *per se* secrete progesterone (Guo *et al.* 2009). The role of endogenous progesterone in the regulation of the induction of PLA2G4A mRNA and protein by cortisol was investigated with trilostane. The cells were treated with cortisol (1 μ M) in the presence and absence of trilostane (1 μ g/ml) for 24 h. As human placenta, the major source of progesterone during gestation, produces increasing amount of progesterone toward the end of pregnancy, we examined the effect of high level of exogenous progesterone on the induction of PLA2G4A expression by cortisol by incubating the cells with cortisol (0.1 μ M) in the presence or absence of exogenous progesterone (0.1–10 μ M) for 24 h. After removal of the medium, total RNA and protein were extracted from the cells for the measurements of PLA2G4A mRNA and protein levels.

Statistical analysis

All data are reported as mean $\pm$ S.E.M. One-way ANOVA test followed by the Student–Newman–Keuls test was used to assess significant differences. Significance was set at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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