Prostaglandin E₂ regulation of amnion cell vascular endothelial growth factor expression: relationship with intramembranous absorption rate in fetal sheep

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AMNIOTIC FLUID (AF) IS A DYNAMIC reservoir with a rapid turnover in late gestation due to high inflows and outflows. The primary amniotic inflows are fetal urine and lung liquid, while the outflows include fetal swallowing and intramembranous (IM) transport of AF across the amnion into underlying fetal blood vessels (4, 20, 23). Fetal urine production, lung liquid secretion, and swallowing have been well described and are regulated by fetal endocrine and nervous systems. The remaining major pathway, IM absorption, has been suggested as the key regulator of AF volume under a variety of experimental conditions (1, 6, 19, 34, 35), but the regulation of IM absorption is currently poorly understood.

Previous studies have shown that the primary component of IM absorption is a unidirectional vesicular transcytosis of amniotic water and solutes across the amnion independent of osmotic and solute concentration differences. A secondary component is bidirectional and dependent on osmotic and solute concentration differences between AF and fetal blood (1, 5, 22). Vascular endothelial growth factor (VEGF) has been shown to activate vesicular transcytosis in vascular endothelial cells (10). Because the gene expression of VEGF in the amnion is elevated when IM absorption rate is increased above normal under various experimental conditions (12, 15, 19, 29), VEGF may similarly mediate IM transport across the amnion. However, the factors that induce VEGF gene expression in the amnion have not been determined.

We recently demonstrated that fetal urine contains a substance that stimulates IM absorption in vivo (1). The identity of this substance has not been elucidated, although it was shown not to be a major solute such as sodium or chloride, nor was it dependent on urine osmolality (1). Fetal urine contains various proteins, endocrine factors, and cytokines in addition to the major solutes. One possible candidate for the unidentified renal derived IM stimulator is the eicosanoid prostaglandin E₂ (PGE₂). This possibility is supported by several observations. Casey et al. (7) found high concentrations of PGE₂ in human fetal urine and concluded that fetal urine is a source of prostanoids in AF. Further, Mitchell et al. (32) reported that human AF contains a fetal urine-derived substance that stimulates PGE₂ production in cultured amnion cells and suggested that “Such a process may be of fundamental importance in the regulation of amniotic fluid volume homeostasis.”

Interactions of PGE₂ and VEGF have been reported under a variety of experimental conditions. PGE₂ or COX2 activation has been shown to increase VEGF gene expression in various cell types (11, 18, 26, 33, 38). In addition to its angiogenic properties, VEGF is a potent cell permeability factor that stimulates transcellular transport via caveolae (10, 21). In vascular endothelial cells, VEGF mediates its effect by down-regulating the expression of caveolin-1, the caveolae structural protein (28). Further, caveolin-1 may function as a negative regulator of VEGF receptor 2 (KDR) activity (27). We have previously shown that VEGF and its KDR receptor are expressed in the ovine amnion (2, 3). Further, in fetal sheep subjected to 4 days of hypoxic hypoxia, VEGF gene expression in amniotic epithelial cells was upregulated (29), while caveo-

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the fetal membranes to enhance AF transport across the amnion by a caveolae-mediated vesicular process.

To explore the potential role of PGE₂ in regulating IM absorption and thus AF volume, we first determined the effects of PGE₂ on VEGF and caveolin-1 gene expression in cultured ovine amnion cells. Second, we analyzed the relationship between amniotic PGE₂ concentration, IM absorption rate, and AF volume in ovine fetuses under conditions of normal, low, or high IM absorption rates. We hypothesized that PGE₂ induces VEGF gene expression in amnion cells leading to downregulation of caveolin-1 expression. We further hypothesized that, in the ovine fetus, PGE₂ concentration in AF correlates positively with IM absorption rate and, therefore, negatively with AF volume, presumably through stimulation of the VEGF-mediated transcytotic pathway in the amnion.

MATERIALS AND METHODS

Ethical approval. These studies were approved by our Institutional Animal Care and Use Committee. We followed the National Research Council’s Guide for the Care and Use of Laboratory Animals.

Amnion cell culture and PGE₂ experiments. Fresh amnion tissues were obtained from late-gestation sheep [129 ± 1 (SE) days gestation, term = 145–150 days] with single or twin pregnancies. Amnion cells were prepared from minced amniotic membranes using three sequential treatments of 0.625% trypsin, as previously described (16). The final cell pellet was resuspended in DMEM/F12 supplemented with 10% FBS and antibiotics, plated onto 75 mm² culture flasks, and were obtained from late-gestation sheep [129 following: for caveolin-1, forward, 5'-GTCTGGCTTCAGGGTG-3' and reverse, 5'-GTCTGGCTTCAGGGTG-3'. The reaction, optimized for efficiency and linearity, was carried out for 28 cycles of 55°C for 30 s and 72°C for 30 s. The amplified products were separated in 2% to 3% agarose gel and visualized with ethidium bromide staining. The intensity of the signal was quantified under ultraviolet light and analyzed by ChemiImager 4400 software (Alpha Innotech, San Leandro, CA).

Western immunoblotting for caveolin-1 protein. Protein lysates were obtained from amnion cells using a nondenaturing lysis buffer and quantified by the protein assay kit (Pierce, Rockford, IL). The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes, as described previously (9, 17). The blots were exposed to rabbit polyclonal anti-human caveolin-1 antibody (N-20, sc-894, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200,000 dilution. The secondary antibody used was a goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). The caveolin-1 protein was visualized with ChemiGlo substrate (Alphalnnotech) using the ChemiImager 4400 (Alpha Innotech). For internal reference, the membrane was stripped and reprobed for β-actin using a mouse monoclonal anti-human β-actin antibody (Santa Cruz Biotechnology) at 1:200 dilution. The intensity of the caveolin-1 signal was referenced to the β-actin signal.

Animals and surgical preparations. Eleven near-term pregnant sheep with singleton fetuses were surgically prepared, as previously described (34). Briefly, a carotid artery catheter was placed for monitoring blood gas status. Fetal urinary bladder and tracheal catheters were placed for sampling and flow rate measurements. A flow probe (Transonic Systems, Ithaca, NY) was placed on the midcervical esophagus for measuring swallowed volume. Multiple catheters were attached to the fetal skin for AF sampling and returning urine and lung liquid to the AF. In vivo data from some of these animals have been presented in part (1, 6). The present study reports new data on PGE₂ concentrations and relationships with AF volume and IM absorption rates.

Fetal sheep experimental methods. Experiments were conducted 5 or more days after surgery at a gestational age of 130 ± 2 days. Fetuses were subjected to 2 or 3 successive 2-day experimental protocols in random order: 1) a control period with monitoring only (n = 11); 2) continuous diversion of fetal urine to the exterior and isovolumic replacement with lactated Ringer solution (n = 11); and 3) AF volume expansion by continuous intra-amniotic infusion of lactated Ringer at 2 μl/day (1.4 ml/min, n = 6). These conditions were chosen to experimentally alter AF volume and IM absorption rates, while modifying amniotic PGE₂ concentrations within the physiological range. Alternative methodologies, such as indomethacin infusion to reduce PGE₂ production or PGE₂ infusion were avoided because of the associated conditions of ductus arteriosus constriction, fetal hypoxia, and reduction in urine production (8, 36).

At the initiation of each experimental protocol, the AF compartment was emptied by drainage for measurement of AF volume and replaced with 1 liter of warm lactated Ringer solution, such that every protocol began with the same volume and composition of AF (1, 34). During each 2-day protocol, fetal swallowing, urine flow rate, and lung liquid production rate were continuously monitored (34). At the end of the 2-day protocol, samples of AF, fetal urine, and fetal lung liquid were collected for measurement of PGE₂ concentration. PGE₂ concentrations in fetal plasma were not determined, as PGE₂ levels in fetal blood are much lower (less than half) than that in AF (37) and would not have significantly impacted the PGE₂ pool in the amniotic compartment. Prior to AF sampling, 20–30 ml of AF were withdrawn from the sampling catheter before collection to ensure a mixed, representative AF sample was obtained. Fetal arterial blood was sampled for blood gases and pH measurements (Radiometer model 725 analyzer; Westlake, OH). AF volume was measured by drainage, and the IM absorption rate was calculated from the time-
integrated amniotic inflows, outflows, and change in AF volume, as previously described (34).

Prostaglandin E₂ determinations. Samples of AF, fetal urine, and lung liquid were subjected to centrifugation (AF, 1,800 g for 15 min; fetal urine, 3,000 g for 10 min; lung liquid, 1,500 g for 10 min) and filtered through 100-μm filters to remove particulates and precipitants. The filtered fluid samples were quantified for PGE₂ concentration by ELISA (PGE₂ EIA kit; Cayman Chemical, Ann Arbor, MI). The inter-assay coefficient of variation was 8.4%, and the ED₅₀ averaged 50 pg/ml. Cross-reactivity of the monoclonal antibody for prostaglandin metabolites was less than 1%. Extraction of PGE₂ from the fluid samples prior to analysis was not performed because our initial analyses indicated that there was minimal interference of the standard values in the presence of filtered amniotic fluid or fetal urine. In addition, extraction of lung liquid samples using a PGE₂ affinity column kit (Cayman Chemical) yielded PGE₂ values comparable to the nonextracted samples. The values of extracted and nonextracted lung liquid samples differed by a mean of 16%, and the difference was not statistically significant by paired t-test.

Calculations, data presentation, and statistical analysis. VEGF and caveolin-1 mRNA levels are expressed relative to their respective 18S reference. Caveolin-1 protein amounts are expressed relative to the respective β-actin levels. Protein and mRNA levels were normalized to the respective values in control group with serum-replaced medium (SR) at 3 h of incubation. Urinary PGE₂ excretion rate was calculated as the product of urine flow rate averaged over each 2-day protocol and urinary PGE₂ concentration. Lung PGE₂ secretion rate was similarly calculated. The data were analyzed by one- and two-factor ANOVA or analysis of covariance (ANCOVA). When the ANCOVA found no difference, the data were combined. For the ANOVA, post hoc comparisons were made using Fisher’s least significant difference for multiple comparisons when the null hypothesis was rejected. Least squares regression analyses were used to characterize relationships between variables. Values were logarithmically transformed to normalize variances as needed. Data are presented as the means ± SE. A P value of 0.05 or less was considered significant.

RESULTS

PGE₂ effects on VEGF gene expression in amnion cells. In amnion cells incubated with control medium containing serum replacement (SR), VEGF mRNA levels were unchanged over time (Fig. 1A). At PGE₂ concentrations of 0.1 μmol/l and lower, VEGF mRNA levels were not significantly altered. With increasing concentrations of PGE₂, there was a significant increase in VEGF mRNA levels (P < 0.0001, Fig. 1A). PGE₂ at concentrations of 1 μmol/l and higher increased VEGF mRNA levels by 3 h (compared to level in the SR group at the same time point). At PGE₂ concentrations of 10 and 100 μmol/l, the increases in VEGF mRNA were both time- (P < 0.05) and concentration- (P < 0.01) dependent, reaching peak levels with 100 μmol/l at 24 h. The VEGF response declined at 48 h of treatment.

PGE₂ effects on caveolin-1 expression in amnion cells. Under control conditions, caveolin-1 mRNA levels did not change over time (Fig. 2A). In response to PGE₂ treatment at 1, 10, and 100 μmol/l, caveolin-1 mRNA levels in amnion cells decreased significantly compared with the SR group at 3 h, and the effects were time- (ANOVA P < 0.01) and concentration- (ANOVA, P < 0.01) dependent (Fig. 2A). Maximal effect was achieved at PGE₂ concentration of 100 μmol/l after 48 h of treatment. The decrease in caveolin-1 mRNA in the presence of increasing PGE₂ concentrations was paralleled by both time- and concentration-dependent reductions in caveolin-1 protein levels (P < 0.05, Fig. 3).

Following treatment of amnion cells with a VEGFR-2 neutralizing antibody at 100 ng/μl, PGE₂ similarly increased VEGF mRNA levels (ANOVA, P < 0.0001, Fig. 1B). At 1 and 10 μmol/l PGE₂, the responses were not different from those in the absence of antibody pretreatment. At 100 μmol/l, the PGE₂-induced increase in VEGF mRNA levels was moderately suppressed (ANOVA, P < 0.05) during neutralizing antibody treatment. In contrast, the PGE₂-induced decrease in caveolin-1 mRNA levels was completely abolished in the presence of VEGFR-2 blockade at all PGE₂ concentrations tested over the 48-h experimental period (Fig. 2B).

In vivo fetal sheep status. During the 2-day control period, fetal arterial pH, carbon dioxide tension, and oxygen tension averaged 7.347 ± 0.006, 53.8 ± 1.3 mmHg, and 22.4 ± 0.8 mmHg, respectively. These values were unchanged during urine replacement and intra-amniotic fluid infusion.

Amniotic fluid, fetal urine, and lung liquid PGE₂ concentrations. During the control period, PGE₂ concentration in the AF was 887 ± 111 pg/ml, significantly lower than 1,843 ± 256 pg/ml in fetal urine but higher than 113 ± 24 pg/ml in fetal lung liquid (ANOVA, P < 0.001). Fetal urine flow rate was 1,142 ± 108 ml/day, and the calculated renal PGE₂ excretion rate was 2,086 ± 254 ng/day. Lung liquid flow rate was 403 ± 67 ml/day, and the calculated lung PGE₂ secretion rate was 44 ± 10 ng/day. The lung PGE₂ secretion rate was equivalent to 4.8% ± 3.1% of the amount of PGE₂ excreted by the kidneys.

Fig. 1. Time- and concentration-dependent vascular endothelial growth factor (VEGF) mRNA responses to prostaglandin E₂ (PGE₂) applied to ovine amnion cells in culture (means ± SE). Cells were grown to 90% confluence in complete medium and incubated in serum-replaced medium (SR) overnight prior to addition of PGE₂. Data were analyzed by ANOVA. *P < 0.05 by post hoc test compared with cells in SR at 3 h of incubation in the absence (A) and in the presence (B) of VEGF-2 neutralizing antibody (100 ng/μl). Time dependence was significant only in the absence of inhibitor (A) at the 2 highest PGE₂ concentrations.
or urinary PGE2 concentrations (AF = 1,190 ± 603 pg/ml; urine = 2,355 ± 545 pg/ml). Similarly, fetal lung liquid PGE2 concentration (104 ± 21 pg/ml) was not affected by intra-amniotic fluid infusion.

**Amniotic fluid and urinary PGE2 concentration relationships.** Fetal urine and amniotic PGE2 concentrations were positively correlated during control conditions and during intra-amniotic infusion. The urinary and amniotic PGE2 concentrations remained positively correlated when urine was diverted and replaced, even though fetal urine did not enter the AF. When the three groups were compared, there were statistically significant shifts in the relationships between amniotic and urinary PGE2 concentrations (ANCOVA, $P < 0.05$, Fig. 4). These changes were due to decreases in amniotic PGE2 concentrations during urine replacement, resulting in a downward shift in the regression line (ANCOVA, $P < 0.01$ compared with control). In contrast, during intra-amniotic infusion, neither amniotic PGE2 concentrations nor the relationship between AF and urinary PGE2 concentrations were different from the control (ANCOVA, $P = \text{ns}$). No correlation was found between amniotic and lung liquid PGE2 concentrations or secretion rates under any of the three experimental conditions.

**Amniotic fluid volume, intramembranous absorption rate, and amniotic PGE2 concentration relationships.** Under control conditions, IM absorption rate (963 ± 118 ml/day) and AF volume (776 ± 134 ml) were negatively correlated ($r = -0.61$, $P < 0.05$). During urine diversion and replacement, AF volume doubled ($P < 0.001$), and IM absorption rate decreased by 57% ± 11% ($P < 0.001$). However, the negative relationship between AF volume and IM absorption rate ($r = -0.58$, $P < 0.01$ for the combined urine replacement and control groups) was not altered (ANCOVA, $P = 0.25$). During intra-amniotic fluid infusion, AF volume and IM absorption rate both increased significantly and remained negatively correlated ($r = -0.96$, $P < 0.01$). However, in response to fluid infusion, the relationship between AF volume and IM absorption rate shifted upward and to the right due to the large increases in both AF volume and IM absorption rate (ANCOVA, $P < 0.0001$, Fig. 5).

IM absorption rate and amniotic PGE2 concentration were not significantly correlated under each of the three experimental conditions (Fig. 6A). Similarly, AF volume and amniotic PGE2 concentration were not significantly correlated (Fig. 6B). Further, the changes in neither IM absorption rate nor AF volume doubled ($P < 0.001$). However, the negative relationship between AF volume and IM absorption rate ($r = -0.58$, $P < 0.01$ for the combined urine replacement and control groups) was not altered (ANCOVA, $P = 0.25$). During intra-amniotic fluid infusion, AF volume and IM absorption rate both increased significantly and remained negatively correlated ($r = -0.96$, $P < 0.01$). However, in response to fluid infusion, the relationship between AF volume and IM absorption rate shifted upward and to the right due to the large increases in both AF volume and IM absorption rate (ANCOVA, $P < 0.0001$, Fig. 5).

IM absorption rate and amniotic PGE2 concentration were not significantly correlated under each of the three experimental conditions (Fig. 6A). Similarly, AF volume and amniotic PGE2 concentration were not significantly correlated (Fig. 6B). Further, the changes in neither IM absorption rate nor AF
sheep urine (1). Our study demonstrated that PGE2 not only function as the stimulator of IM absorption present in fetal caveolin-1 gene expression in amnion cells and potentially prostanoid abundant in fetal urine, would alter VEGF and sized, but also that the PGE2 effect on caveolin-1 expression in ovine amnion cells in vitro, as hypothe-
ted. A comparison of the relationships between amniotic fluid volume and intramembranous absorption rate by ANCOVA. • denotes control conditions, ○ denotes fetal urine replacement, and ■ denotes continuous 2 l/day intra-amniotic infusion of lactated Ringer solution. Data from control and urine replacement groups were combined prior to analysis because the regression relationship between these two groups was unaltered by urine replacement (ANCOVA, \( P = 0.25 \)).

volume were correlated with the changes in amniotic PGE2 concentration. When analyzed collectively, the regression relationship between IM absorption rate and amniotic PGE2 concentration shifted significantly downward with urine replacement and upward during intra-amniotic fluid infusion (ANCOVA, \( P < 0.001 \), Fig. 6A). These changes were due to alterations in IM absorption rate independent of PGE2 concentration. Comparable results were obtained by analysis of the relationships between AF volume and amniotic PGE2 concentrations (ANCOVA, \( P < 0.001 \), Fig. 6B). The upward shifts in the regression lines for the urine replacement and infusion groups were due to changes in AF volume independent of PGE2 concentration. These results were not altered by either exclusion of the one outlier with high amniotic PGE2 concentrations in Fig. 6 or by log transformation of the data (after excluding the one negative IM absorption rate in Fig. 6A).

When analyzed against renal PGE2 excretion rate rather than amniotic PGE2 concentration, similar results were obtained in that neither IM absorption rate nor AF volume were correlated with PGE2 excretion rate during control conditions or during intra-amniotic fluid infusion (data not shown). As expected, there were no significant correlations during urine diversion and replacement when urine did not enter the AF. There was no significant correlation between IM absorption rate or AF volume and lung liquid PGE2 concentrations under any of the experimental conditions studied.

**DISCUSSION**

An objective of this study was to explore whether PGE2, a prostanoid abundant in fetal urine, would alter VEGF and caveolin-1 gene expression in amnion cells and potentially function as the stimulator of IM absorption present in fetal sheep urine (1). Our study demonstrated that PGE2 not only upregulated VEGF gene expression and downregulated caveo-
lin-1 expression in ovine amnion cells in vitro, as hypothe-
sized, but also that the PGE2 effect on caveolin-1 was mediated through the VEGFR-2 receptor. These observations are consist-
tent with the established role of VEGF in stimulating vesicular transcytosis via caveolae (10), and in downregulating caveo-
lin-1 expression observed in vascular endothelial cells (28).

Further, these findings support our earlier report that increases in IM absorption induced by hypoxia was associated with elevated VEGF but reduced caveolin-1 gene expression in the amnion (15).

Our second objective was to examine the relationship be-
tween amniotic PGE2 concentrations and IM absorption rate in chronically catheterized ovine fetuses under experimental conditions of normal, low, or high IM absorption rates. On the basis of our in vitro findings in amnion cells, we expected that amniotic PGE2 concentrations would correlate positively with IM absorption rate in vivo, presumably through the activation of VEGF-mediated transcytotic pathways. However, our present studies in ovine fetuses do not support the functionality of such a pathway because neither amniotic PGE2 concentration, urinary PGE2 concentration, nor renal PGE2 excretion rate correlated with IM absorption rate or AF volume under control and experimentally induced conditions of decreased or increased IM absorption. The lack of relationship likely is due to the in vivo concentrations of PGE2 in AF (250–1,000 pg/ml or 1–4 nmol/l) being lower than the effective dose of PGE2 in the in vitro amnion cell studies (1,000–100,000 nmol/l). Thus, under normal physiological conditions, amniotic PGE2 would not be involved in the maintenance of IM transport and, thus, of AF volume. However, participation of PGE2 in the regulation of AF dynamics may occur under adverse conditions when amniotic PGE2 is greatly elevated, such as during intrauterine infection when fetal and amniotic PGE2 concentrations reach ~20 times normal in pregnant sheep (24) and ~120 times normal in Rhesus monkeys (25).

In addition to our original objectives, the present study has provided several unique observations regarding PGE2 levels in...
the AF compartment. In near-term ovine fetuses under normal conditions, amniotic PGE₂ concentrations are high, while urinary levels are twice amniotic levels. The fetal kidneys normally excrete more than 40 times as much PGE₂ as the fetal lungs secrete into the AF. With high renal excretion rates, it is not unexpected that a positive correlation existed between urinary and amniotic PGE₂ concentrations under control conditions and during intra-amniotic fluid infusion. These results are consistent with the notion that fetal urinary PGE₂ is a primary contributor to the PGE₂ pool in AF. However, the observation that amniotic and urinary PGE₂ concentrations remained positively correlated but shifted downward when fetal urine was replaced with Ringer solution was not expected and suggests that amniotic PGE₂ and urinary PGE₂ levels are regulated by a common endogenous factor presumably present in the fetal circulation. This observation requires further investigation.

Because fetal urine contributes about 58% to the amniotic PGE₂ pool, while lung liquid contributes 3%, the remaining PGE₂ in AF must be derived from other sources. Because PGE₂ concentration in ovine fetal blood is low compared with AF concentrations (37), the fetal vascular system would be a sink rather than source of amniotic PGE₂. The most likely source would be endogenous production by the fetal membranes and, to a lesser extent, the umbilical cord (30, 31). Our finding that amniotic PGE₂ concentration during urine replacement was much higher than could be explained by a pulmonary source alone also supports this possibility.

**Perspectives and Significance**

Although it has long been recognized that AF volume is regulated by the rate of IM absorption, presently there is no detailed knowledge of the factors involved in modulating IM transport across the amnion and into the underlying fetal vasculature. Over the past decade, the most significant advance in this understanding has been the observations that IM transport across the amnion has two components: a primary unidirectional vesicular transcytosis of AF mediated by VEGF and a secondary passive bidirectional diffusion of water and solutes down concentration and osmotic gradients between AF and fetal blood through aquaporin water channels. The recent observation that fetal urine contains a stimulator of IM absorption (1) raised the possibility that PGE₂ is the unidentified renal stimulator of IM absorption across the amnion. This is because PGE₂ is present in fetal urine in high concentrations, and PGE₂ is known to stimulate cellular VEGF expression and function. However, in contrast to our hypothesis, PGE₂ does not appear to be involved in the regulation of AF volume under physiological conditions. Thus, the stimulator of IM absorption present in fetal urine remains to be identified.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


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