Prostacyclin is an autocrine regulator in the contraction of oviductal smooth muscle

Farinaz Arbab1, Jennifer Goldsby2, Nena Matijevic-Aleksic3, Gangxiong Huang3, Ke-He Ruan3 and Jaou-Chen Huang2,4

1Department of Pathology, Baylor College of Medicine, 2Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology and 3Vascular Biology Center Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center at Houston, Houston, Texas, USA

4To whom correspondence should be addressed at: Department OB/GYN, UT Medical School, 6431 Fannin Street, MSB 3.604, Houston, TX 77030, USA; E-mail: Jaou-Chen.Huang@uth.tmc.edu

BACKGROUND: It was recently discovered that prostacyclin constituted 40–50% of prostaglandins (PG) produced by minced human oviduct. It is well established that prostacyclin relaxes vascular smooth muscle, but whether oviductal smooth muscle synthesizes prostacyclin and whether its contraction is affected by prostacyclin remain unclear. METHODS: Smooth muscle microdissected from human oviducts was used for the study. The expression of prostacyclin synthase (PGIS) and prostacyclin receptor (IP) was confirmed by Western blot analysis. Metabolites of [3H]PGH2 were analysed for prostacyclin. Functional coupling of IP to adenyl cyclase was assessed by the accumulation of intracellular cAMP upon prostacyclin challenge. The presence of saturable, specific binding sites for prostacyclin was confirmed by binding assay. The identity of IP was further confirmed by RT–PCR and nucleotide sequence analysis. Finally, the effects of prostacyclin on muscle contraction were studied. RESULTS: Human oviductal smooth muscle expresses functionally active PGIS and IP. The IP expressed is the same as that cloned from human lung tissue. The ED50 of prostacyclin to increase intracellular cAMP was 16 nmol/l. Prostacyclin dose-dependently decreased the amplitude of muscle contraction. CONCLUSIONS: Human oviductal smooth muscle produces prostacyclin, which, in turn, decreases its contractility. Prostacyclin may regulate embryo transport.

Keywords: cAMP/embryo transport/prostacyclin receptor/prostaglandins

Introduction

After fertilization, the embryo begins its journey from the distal end of the oviduct to the uterus (Speroff et al., 1999). Transport of the embryo is aided by cilia inside the oviduct as well as by the smooth muscles of the oviduct (Vizza et al., 1995). The contractility of oviductal smooth muscle is regulated by many factors including β-adrenergic nerves (Helm et al., 1982), sex steroids (Maia and Coutinho, 1970; Lindblom et al., 1980; Helm et al., 1982; Nozaki and Ito, 1986), nitric oxide (Ekerhovd et al., 1997, 1999; Perez Martinez et al., 2000) and prostaglandins (PGs) (Lindblom et al., 1978, 1979, 1980; Wilhelmsson et al., 1979). It has been shown that different PGs have distinct effects on the contractility of oviductal smooth muscle in vitro (Lindblom et al., 1978, 1983). While PGE2 contracts the muscle, the responses elicited by PGE2 depend on the concentrations of PGE2 and/or types (circular or longitudinal) of muscle studied (Lindblom et al., 1978). Earlier reports regarding the effects of prostacyclin on oviductal muscle were not conclusive: one showed biphasic responses induced by prostacyclin (Omini et al., 1978), while the other showed contraction in the longitudinal muscle and minimal relaxation in the circular muscle (Lindblom et al., 1979). The biosynthesis of PGs involves the conversion of arachidonic acid to PGG2 and PGH2, catalysed by prostaglandin H synthase (PGHS, also called cyclo-oxygenase, COX) (Smith and Marnett, 1991). The conversion of PGH2 to each PG is catalysed by the individual isomerase: prostacyclin synthase (PGIS) catalyses the conversion of PGH2 to prostacyclin, and prostaglandin E synthase to PGE2, etc. PGHS has been reported in human oviduct (Van Voorhis et al., 1990), as was the synthesis of PGE2 and PGF2α (Ogra et al., 1974). However, there is no conclusive evidence to suggest that human oviduct synthesizes prostacyclin (Lindblom et al., 1979). Similarly, the expression of PGIS in the oviductal smooth muscle has not been reported.

Recently, it was discovered that 40–50% of the PGs synthesized by minced human oviduct was prostacyclin (Tumbusch et al., 2001). This prompted an investigation of the synthesis of prostacyclin and expression of PGIS by smooth muscle of the oviduct. The well-documented relaxation effect of prostacyclin on the vascular smooth muscle also prompted a search for a prostacyclin receptor (IP) in the oviductal smooth muscle and an investigation into the effects of prostacyclin on muscle contraction. As a second IP had been reported in the...
brain (Watanabe et al., 1999), a comparison was also made between the IP expressed by the oviductal smooth muscle and that cloned previously from the lung (Boie et al., 1994).

Materials and methods

Materials

Unless specified otherwise, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Segments of Fallopian tubes were obtained from patients undergoing gynaecological surgery for benign conditions unrelated to the oviduct. Samples were placed on ice and, after immediate transfer to the laboratory, were either used immediately or stored at −70°C until future use (see below). This project was reviewed and approved by the institutional review board of the authors’ institution.

Preparation of microsomes

Smooth muscle was microdissected from the oviducts and homogenized using a tissue homogenizer (Tissumizer, Tekmar Co., Cincinnati, OH, USA) in a buffer containing protease inhibitors (1 mmol/l 4-(2-amino-ethyl) benzene sulphonyl fluoride hydrochloride, 0.8 µmol/l aprotinin, 50 µmol/l betagastatin, 15 µmol/l LE-64, 20 µmol/l leupeptin hemisulphate, 10 µmol/l pepstatin A; Calbiochem-Novabiochem Corp., San Diego, CA, USA). The homogenate was first centrifuged (10 000 g) at 4°C for 10 min; the supernatant was removed and further centrifuged (100 000 x g) at 6°C for 50 min. The pellet was homogenized in a resuspension buffer (50 mmol/l Tris–HCl pH 8.0, 2 mmol/l EDTA, 1 mmol/l diethylthiocarbamate, 2.45 mmol/l l-tryptophan) using a hand-held tissue homogenizer. Protein concentrations of the microsome suspension were determined using bovine serum albumin (BSA) as the standard (Micro BCA, Pierce Chemical Co., Ann Arbor, MI, USA).

Metabolism of [3H]PGH2

Metabolites of [3H]PGH2 (specific activity 209 µCi/µmol; Cayman Chemical, Ann Arbor, MI, USA) were analysed by reverse-phase high-pressure liquid chromatography (HPLC) according to a procedure described previously (Sanduja et al., 1991). Briefly, microsomes from oviductal smooth muscle (68.5 µg) were resuspended in 100 µl incubation buffer (50 mmol/l Tris–HCl pH 8.0, 2 mmol/l EDTA, 1 mmol/l glutathione, 1 mmol/l l-tryptophan) containing 20 µmol/l H2PGH2 and incubated at 37°C for 30 min. The supernatant was extracted (Sep-Pak cartridges C18, Waters Corp., Melford, MA, USA). The eicosanoids were separated by HPLC (Waters) and detected using an in-line radio-detector (β-Ram, Inus Systems Inc., Tampa, FL, USA). The acquired data were analysed using Millenium 32® software (Waters). The retention time of each PG has been determined previously using individual standards. For the negative control, i.e. the non-enzymatic conversion of PGH2, phosphate-buffered saline (PBS) was used in place of microsomes.

Culture of muscle cells

The culture of muscle cells was based on methods to culture stromal cells from the endometrium (Huang et al., 1998) with some modifications. Smooth muscle was microdissected from the oviduct, minced into 0.5-mm pieces and placed in DMEM/F-12 media containing collagenase (1000 units/ml; Worthington Biochemical Corp., Freehold, NJ, USA), DNase (1000 U) and 2% chick serum. The mixture was shaken in a 37°C waterbath for 30 min. The pellet of the mixture, containing cells and incompletely digested muscle pieces, was maintained in DMEM/F12 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (68.6 µmol/l), fungizone (0.54 µmol/l) and insulin (0.25 units/ml). After 7–10 days, non-viable tissues were removed and the attached cells were maintained in the same media until reaching confluence. The purity of the cultured smooth cells was confirmed by immunohistochemical staining using monoclonal antibody against smooth muscle actin. More than 99% of cells were smooth muscle cells. Cells from the first or the second passage were subcultured in 10 cm, 6-well or 96-well plates for experiments.

Western blot analysis

The Western blot analysis was performed according to methods described previously (Huang et al., 1998). Aliquots of microsome suspension (20 µg each) from smooth muscle of the oviduct or cultured smooth muscle cells were separated using a 10% acrylamide gel and transferred to a 0.45 µm nitrocellulose membrane (Scheicher and Schuell, Keene, NH, USA). Affinity-purified rabbit polyclonal antibody against peptide sequences of human PGIS (Deng et al., 2002) and IP were used to detect the respective protein. Lysates of Cos-7 cells transfected with a vector expressing human PGIS and IP (created by K.-H.Ruan) were used for positive controls. Enhanced chemifluorescence (Amersham Biosciences Corp., Piscataway, NJ, USA) was used for visualization; the fluorescence signals were detected using a laser scanner (STORM 850, Molecular Dynamics, Sunnyvale CA, USA).

RT-PCR and sequence analysis

Total RNA was extracted from cultured smooth muscle and WI-38 cells (a diploid fibroblast cell line from human lung tissue; ATCC, Manassas, VA, USA) using a commercial kit (Qiagen, Chatsworth, CA, USA). Total RNA (3 µg) was reverse-transcribed at 42°C for 50 min using oligo dT12-18 and Superscript II RT in 20 µl reverse-transcription mixture (Invitrogen, Carlsbad, CA, USA). An aliquot of the cDNA (2 µl) was used for PCR. Oligo primers based on human IP sequences 67–87 (exon 2) and 1246–1224 (exon 3) (Genebank NM_000960) were used to amplify the cDNA. The 50 µl aliquot of PCR reaction buffer contained 20 µmol of each dNTP, 1 µl cloned pfu DNA polymerase, 0.4 µmol of each primer and 2 mmol/l MgCl2 (Stratagene, La Jolla, CA, USA). The PCR consisted of one cycle at 95°C for 2 min, 30 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 2 min, and a final extension of 72°C for 10 min. A 10 µl portion of the PCR product was separated by 1% agarose gel electrophoresis. After staining with ethidium bromide, the DNA was visualized using ultraviolet light. The molecular weight of the product was estimated using a 1 kb DNA ladder (Promega Co., Madison, WI, USA). The nucleotide sequence and deduced amino acid sequence were compared with that of IP cloned from human lung tissue (Genebank NM_000960).

Muscle contractility experiments

Muscle strips from longitudinal and circular layers of the oviduct were dissected from the ampullo-isthmic junction, identified by advancing a blunt probe (1.5 mm) from the fimbrial end until resistance was met (Lindblom et al., 1978). The strips were placed in organ chambers according to methods described previously (Weisbrodt et al., 1985). The chambers were filled with Krebs–Ringer solution (NaCl 136 mmol/l, KCl 4.7 mmol/l, CaCl2 2.5 mmol/l, MgCl2 1.5 mmol/l, NaH2PO4 1.8 mmol/l, NaHCO3 31.5 mmol/l, glucose 15 µmol/l), heated to 37°C and oxygenated with 95% O2 and 5% CO2. The muscle strips were placed under a tension equivalent to a load of 1.0 g and attached to isometric transducers. After the pattern of contraction had stabilized (usually in 30 min), increasing amounts of iloprost (a stable analogue of prostacyclin) were added to the chamber at 5-min intervals to attain concentrations from 0.01 nmol/l to 25 µmol/l. Contractility of the muscle was recorded.
on a polygraph recorder (Polygraph Model 7; Grass Instrument, Quincy, MA, USA). On completion of the experiment, viability of the muscle was confirmed by contractions induced by calcium ionophore A23187 (2 µmol/l). The mean (±SD) of the amplitude of contractions was calculated. The amplitude of contraction recorded at baseline was set as 100%, and the responses to iloprost were expressed as percentage amplitude at baseline.

**Whole-cell binding assay**

Muscle cells in 6-well plates (86 000 cells/well) were used for the whole-cell binding assay. After two washes using Hanks’ balanced salt solution (HBSS, calcium- and magnesium-free), one set of the cells was incubated in HBSS (with 5 mmol/l MgCl₂ and 0.1% BSA) containing increasing concentrations of [³H]iloprost to determine the total binding. Another set of cells received, in addition to [³H]iloprost, a 200-fold excess of non-radioactive iloprost to assess non-specific binding. After 2.5 h, the cells were washed twice with cold HBSS containing 0.1% BSA. The cells were lysed and the radioactivity was determined. Specific binding was calculated by subtracting the non-specific binding from the total binding.

**Accumulation of intracellular cyclic AMP**

Muscle cells in 96-well plates (50 000 cells/well) were incubated with iloprost or specified chemical(s) in the presence of isobutyl methyl xanthine (IBMX, 1 mmol/l) for 30 min at 37°C. After incubation, the medium was removed and the cells lysed. The intracellular cAMP content was determined using an enzyme immunoassay according to the manufacturer’s protocol (Amersham).

**Statistical analysis**

The ED₅₀ was calculated using GraphPad Prism Version 3.0 for Windows (San Diego, CA, USA) with the Hill slope set at 1.0.

**Results**

Western blot analysis of microsomes from oviductal smooth muscle (two samples each from follicular and luteal phases of the menstrual cycle) and cultured muscle cells confirmed the presence of PGIS in all samples (Figure 1). The activity of PGIS was verified by analysing the metabolites of [³H]PGH₂.

Aliquots of microsomes converted [³H]PGH₂ to prostacyclin, as confirmed by the presence of its stable metabolite, 6-keto-PGF₁α (Figure 2a). The negative control showed negligible conversion (Figure 2b).

Western blot analyses were then performed to determine the expression of IP in cultured muscle cells and oviductal smooth muscle (two samples each from follicular and luteal phases of the menstrual cycle). All microsomal preparations contained a protein detected by the affinity-purified antibody against human IP. The molecular weight of the protein (~52 kDa) was identical to that of recombinant human IP (Figure 3). The identity of the IP was further confirmed by RT–PCR and nucleotide sequence analysis. RT–PCR of total RNA from cultured smooth muscle cells and WI-38 cells (a fibroblast cell line of human lung, from which the first IP was cloned) yielded amplicons with identical molecular size (Figure 4). Some 99% of the nucleotide sequences were identical, while the deduced amino acid sequences were exactly the same (data not shown).

The functional coupling of IP to adenyl cyclase was confirmed by an accumulation of cAMP after challenging the cells with iloprost, a stable agonist of prostacyclin (Figure 5a). Upon challenge, cultured muscle cells accumulated cAMP in a dose-dependent manner (Figure 5b). The ED₅₀ to increase intracellular cAMP was 16 nmol/l. The presence of specific, saturable binding sites for iloprost in the same cells was confirmed by whole-cell binding assay using [³H]iloprost (Figure 6).

In order to determine the effects of prostacyclin on muscle contraction, the contractions of muscle strips were studied in organ chambers. Both longitudinal and circular muscles displayed spontaneous, rhythmic contractions in the organ chamber, with frequencies of 5 and 8/min respectively. The pattern of contraction stabilized after 30 min, and remained unchanged for more than 8 h. Iloprost decreased the amplitude of contraction (Figure 7a) in a dose-dependent manner (Figure 7b and c). However, there were subtle differences: at
Figure 2. Oviductal smooth muscle expressed functionally active prostacyclin synthase, which converted $[^{3}H]$prostaglandin H$_{2}$ (PGH$_{2}$) to prostacyclin. A representative chromatogram is shown. (a) Microsomes prepared from tubal smooth muscle were incubated with $[^{3}H]$PGH$_{2}$. The supernatant, analysed by high-pressure liquid chromatography, contained 6-keto PGF$_{1\alpha}$, a stable metabolite of prostacyclin. (b) The conversion of PGH$_{2}$ to 6-keto PGF$_{1\alpha}$ by phosphate-buffered saline was negligible. The experiment was repeated using samples from different sources and results were identical. I = 6-keto PGF$_{1\alpha}$; B = thromboxane B$_{2}$, the stable metabolite of thromboxane A$_{2}$; E = PGE$_{2}$; F = PGF$_{2\alpha}$; D = PGD$_{2}$; 12-HETE = 12-hydroxyeicosatetraenoic acid.

25 µmol/l iloprost, the amplitude of contractions was reduced to $37 \pm 0$ and $17 \pm 5\%$ (mean ± SD, based on six contractions) of baseline for longitudinal and circular muscles respectively, while the ED$_{50}$ values were ~400 and ~100 nmol/l respectively. The effects on the frequency of contractions were less dramatic: from 5 to 4/min for longitudinal muscle, and from 8 to 5/min for circular muscle. The findings were confirmed by a separate experiment using muscles from a different source.

Discussion

The results of the present study provide evidence for the presence of functional PGIS and IP in the smooth muscle of human oviduct. These data also suggest that the IP is identical to the first IP cloned from human lung (Boie et al., 1994). The ED$_{50}$ to increase intracellular cAMP in cultured smooth muscle cells (16 nmol/l) was in the physiological range (Tsai et al., 1988) and consistent with the role of a paracrine/autocrine regulator.

These results indirectly confirmed a previous report that cAMP and inhibitors of phosphodiesterase decreased the contractility of oviductal smooth muscle (Lindblom and Hamberger, 1980). Furthermore, the present data are consistent with the signal transduction of IP (Boie et al., 1994) and how cAMP affects calcium mobilization within cells. Upon binding by its ligand, IP increases intracellular cAMP (Boie et al., 1994), which in turn decreases inositol 1,4,5-triphosphate (IP$_{3}$) and increases IP$_{3}$ 3-kinase activity, resulting in lowered intracellular calcium level and decreased muscle tone (Abdel-Latif, 1996; Bolton et al., 1999).
Prostacyclin and IP in smooth muscle of oviduct

Figure 5. Functional coupling of prostacyclin receptor (IP) and adenyl cyclase. When challenged by iloprost, the cultured smooth muscle cell increased intracellular cAMP content in dose-dependent manner. (a) Preliminary experiments using phosphodiesterase inhibitor (isobutyl methyl xanthine, IBMX), adenyl cyclase activator (forskolin) and iloprost (a stable agonist of prostacyclin) confirmed the functional coupling. (b) Cultured muscle cells were challenged by increasing concentrations of iloprost in the presence of IBMX. The figure shows the mean of two independent experiments using cells from different sources. ED$_{50}$ = 16 nmol/l.

Figure 6. Cultured muscle cells expressed saturable binding sites for prostacyclin. [H]iloprost was used in the binding assay. Non-specific and total binding were determined by incubating cells with and without a 200-fold excess of non-radioactive iloprost. Specific binding was obtained by subtracting non-specific binding from total binding.

Although the contractility of muscle was reduced by iloprost, there was a 4-fold difference in the ED$_{50}$ for the longitudinal and circular muscles (~400 versus ~100 nmol/l), though this might be due to different receptor densities or receptors with different binding affinities in the different muscles. A differential response to PGE$_2$ has been reported in

Figure 7. Iloprost decreased muscle contraction in dose-dependent fashion (results from one experiment). (a) Patterns of contractions exhibited by longitudinal and circular muscles at baseline, in the presence of 0.5 µmol/l and 25 µmol/l iloprost. Longitudinal (b) and circular (c) muscles responded differently to iloprost: ED$_{50}$ values to decrease the amplitude of contractions were ~400 nmol/l and ~100 nmol/l respectively. Amplitudes at baseline were 29.1 mm and 16.1 mm for longitudinal and circular muscles respectively. These findings were confirmed by another experiment using muscles from a different source.
oviductal muscles: the ED₉₀ to stimulate the longitudinal muscle was 15 nmol/l, while that to relax the circular muscle was 1.3 nmol/l (Lindblom et al., 1978).

The maximum reduction in the amplitude of contraction achieved with 25 µmol/l iloprost was 63 and 83% that of baseline for the longitudinal and circular muscles respectively. The inability of iloprost to relax the muscles completely might be due to internalization of IP; alternatively, iloprost at supraphysiological concentrations may bind non-specifically to receptors for other PGs, and this may oppose the relaxation effects of iloprost.

The present results differ from those of two earlier reports concerning the effects of iloprost on muscle contractility. One report showed that prostacyclin, at concentrations between 26.7 and 1670 nmol/l, contracted the longitudinal muscle and relaxed the circular muscle (Lindblom et al., 1979); the other showed that prostacyclin had biphasic effects on muscle contraction (Omini et al., 1978). It is speculated that impurities in the prostacyclin might have caused these contractions, or the disparity might have been due to the fact that different muscles were studied. The proportions of receptors for various PGs may vary in different segments of the oviduct. At supraphysiological concentrations, iloprost may bind to IP and, non-specifically, to receptors for other PGs, which may in turn oppose the effects of iloprost.

Compared with the degree of reduction in the amplitude, the reduction in frequency was less dramatic. The present observations concur with those reported previously when prostacyclin reduced the amplitude of contraction by 50% without significantly decreasing the frequency of contraction (Lindblom et al., 1979). The pacemaker in the smooth muscle at the ampullo-isthmic junction used in the current study may resemble that of the gut muscle, the interstitial cells of Cajal at the ampullo-isthmic junction used in the current study may resemble that of the gut muscle, the interstitial cells of Cajal—

the activity of which is not affected by cAMP (Horowitz et al., 1999).

The transport of human embryos in the oviduct consists of a relatively quick passage through the distal tube, a delay for 2–3 days in the proximal ampulla, and a rapid transit through the isthmus (Croxatto et al., 1977). Indirect evidence suggests the presence of a physiological sphincter regulated by PGs in the human oviduct (Lindblom et al., 1978). Thus, non-steroidal anti-inflammatory agents may affect embryo transport in the oviduct.

In addition to embryo transport, prostacyclin derived from the muscle may diffuse into the lumen of the oviduct and affect the performance of gametes and development of the embryos. Effects of prostacyclin on non-cardiovascular systems have been underscored by observations on gene knockout mice: prostacyclin has an indispensable role in both embryo implantation (Lim et al., 1999) and pain sensation (Ohishi et al., 1999; Ueno et al., 2000). In addition, one clinical observation suggested that a low seminal prostacyclin level was associated with decreased sperm motility (Schlegel and Meyer, 1986).

In summary, oviductal muscle is both a source and a target of prostacyclin. The IP expressed by the oviductal muscle was identical to the classical IP cloned from human lung, Prostacyclin may regulate embryo transport by relaxing the muscle of the oviduct.

Acknowledgements

The authors thank Ms Mary Gilliland for secretarial support. These studies were supported in part by National Institute of Health grants to K.-H.R. (HL 56712), and to J.-C.H. (a Women’s Reproductive Health Research Scholar, HD 01277).

References


Submitted on 5 June 2002; accepted on 16 August 2002