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# Basic Science

## IN VITRO STUDIES ON THE CONTROL OF HUMAN MYOMETRIAL GAP JUNCTIONS

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### Abstract

*In this study human myometrial tissues were examined for the presence of gap junctions by quantitative electron microscopy before and after incubation in tissue culture media with and without indomethacin. The area of gap junctions was very low in tissues from pregnant women at term but not labor, before incubation. After 24 and 48 h incubation without any treatment, segments of some of the same tissues developed many gap junctions and other tissues contained few junctions. Prostaglandin E (PGE), prostaglandin F (PGF) and prostaglandin F metabolite (PGF metabolite) levels in the media at various times were measured by radioimmunoassay. The prostaglandins increased progressively during the incubation period. Treatment of tissues with indomethacin decreased prostaglandin levels in the media and increased the numbers of gap junctions in those control tissues that developed few junctions over the same incubation interval. We conclude that the capacity of human myometrial tissues to develop gap junctions in vitro may depend upon a maturational stage in preparation for labor. Furthermore, our results suggest that products of the cyclo-oxygenase or lipoxygenase pathways may control the presence of gap junctions in the human myometrium and that changes in synthesis in these patterns may occur as part of the maturational process.*

**Keywords:** Myometrium; Labor; Gap junctions; Prostaglandins; Uterus; Pregnancy.

### Introduction

Gap junctions develop between muscle cells of the myometrium during term and preterm labor in various animals [6,11,17] and humans [11]. The presence of the junctions correspond to the time when the myometrium is active and the occurrence of these cell-to-cell contacts is thought to be responsible for changing the myogenic properties of the muscle cell such that electrical events propagate between cells to synchronize contractility [18]. If gap junctions are necessary for labor then an understanding of the conditions which either stimulate or inhibit their presence is of considerable clinical significance.

The mechanisms which promote the existence of myometrial gap junctions is not completely understood. In animals, changes in the levels of steroid hormones and prostaglandins, which precede and accompany labor, are thought to control the synthesis and enlargement of the junctions [7–10,11]. In the human myometrium there is no evidence for these regulatory mechanisms and the steroid hormone changes which take place before and during labor are not as distinct as those of most animals [2]. How-

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ever, in all species including humans increased synthesis and release of prostaglandins are apparent and these compounds appear to be involved in the onset and progression of labor [1,2,5]. There is also evidence that prostaglandins interact with the steroid hormones to regulate gap junctions in the rat uterus [14]. However, there are conflicting reports as indomethacin treatment appears to inhibit gap junctions in some circumstances [8] and stimulate their presence in others [14]. It is possible that the prostaglandins are the primary control mechanism for gap junction formation in the human uterus.

Studies of some treatments which may prevent or initiate gap junctions are not possible in humans. However, previous studies with animal tissues have shown that gap junctions develop spontaneously in vitro and this model system has been used to investigate agents which either promote or inhibit the formation of junctions [7,8]. In the present study, we quantitated the numbers of gap junctions in myometrial tissues from women not in labor before and with and without treatment with indomethacin. Our study shows that the gap junctions develop slowly in vitro and the results indicate that a product(s) of the cyclooxygenase or lipogenase pathway may control the presence of the junctions.

## Materials and methods

The methods used in this study were similar to those used previously for animal [7,8] and human tissues [11].

*Tissues*

Uterine tissues (approx. 3 × 2 × 1 cm) were excised from the lower uterine segments of the cesarean openings from 12 women undergoing elective operations. The average gestation age was 39 weeks with a range of dates from 36 to 41 weeks. None of the patients was in active labor. Informed consent to remove the tissue and participate in the study was obtained from each woman by the attending physician. This project was approved by the Ethics Committees of the authors' institutions.

Immediately after removal the tissues were placed in ice-cold Krebs-Ringer's solution and within 20 min divided into pieces (approx. 1 cm × 1 mm × 0.5 mm) with the muscle oriented in the long axis. Each piece was stretched 1.5 times the excised length with stainless steel pins. Some tissues were then fixed for electron microscopy (control = zero time). Other tissues from the same uterine segment were fixed after incubation in a $CO_2$ incubator at 37°C for various times and treatments. The incubation media used was MEM (Minimum Essential Medium, Gibco) without fetal calf serum. Samples of the media were collected at selected times and analyzed for prostaglandins. Indomethacin (Merck Frosst Laboratories) was dissolved in 1% sodium carbonate and added to the culture media in 10 μl volumes. The same volume of sodium carbonate was also added to media containing control tissues.

*Electron microscopy*

All tissues were fixed by immersion in buffered 2% glutaraldehyde solution and prepared for electron microscopy as described previously [6–11]. The tissues were examined and photographed (approx. 40 photos/tissue) in a Philips Model 301 electron microscope. The lengths of the gap junctions and nonjunctional membranes were determined in the photographs by the methods used formerly [11].

*Prostaglandin analysis*

The procedures for prostaglandin measurements have also been published previously [12,13] with only minor changes for this study. No extraction or column chromatography was performed for analysis of the samples of tissue culture media. Briefly, the media samples (1 ml) were placed in polypropylene tubes (Falcon, Oxnard, CA) containing enough indomethacin to give a final concentration of at least 10 μg/ml and centrifuged at 500 × *g* for 20 min. The supernatants were

placed in capped tubes and stored at −20°C until assay. The frozen media were then thawed and assayed directly without extraction or chromatography. Two 100-μl aliquots were used for each assay of PGF, PGE and 13,14-dihydro-15-keto PGF (PGF metabolite). To each Biovial® (Beckman Instruments, Fullerton, CA) was added 100 μl sample, 200 μl of phosphate buffered saline containing 0.3% bovine gamma globulin (Miles Laboratories, Inc., Elkhart, IN) and 5500 cpm of the appropriate labelled PG – [5,6,8,9,11,12,14,15(*n*)-$^3$H] $PGF_2$ (178 Ci/mmol), [5,6,8,11,12,14,15(*n*)-$^3$H] $PGE_2$ (178 Ci/mmol) or [5,6,8,9,11,12,14(*n*)-$^3$H] 13,14-dihydro-15-keto $PGF_2$ (80 Ci/mmol) (Amersham Corp., Arlington Heights, IL) – diluted in 100 μl of the same buffer. The tubes were vortexed and 100 μl of the appropriate antibody added. The antibodies were obtained from Seragen, Boston, MA (anti $PGF_2$, batch No. 212205, dilution 1:35000: anti $PGE_2$, batch No. 301132, dilution 1:14000) or Dr. K. Kirton, Upjohn Co., Kalamazoo, MI (anti PGF metabolite, batch No. 11560-JCC-140D, 80 mg/ml diluted 1:25000 for assay). The cross-reactions of these antibodies were as follows. Anti $PGF_2$: $PGF_2$, $PGF_1$ – 100%; $PGE_1$, 6-keto $PGF_1$ – 1.1% and all others less than 0.5%. Anti $PGE_2$: $PGE_2$, $PGE_1$ – 100%; $PGA_2$ – 6%; $PGA_1$ – 3%; 6-keto $PGE_1$ – 1%; $PGF_2$ – 1.3% and all others less than 1%. Anti PGF metabolite: 13,14-dihydro-15-keto $PGF_2$ – 100%; 15-keto$_2$ – 20%; C-16 urinary metabolites of $F_2 \leqslant 1\%$; 13,14-dihydro $PGF_2$ – <0.5% and $PGF_2$ – 0.1%. This 1st antibody was very kindly provided by Dr. K.T. Kirton of the Upjohn Co. and the data regarding cross-reactions are taken from a personal communication from him.

After addition of the antibody, the tubes were incubated for 4 h at 4°C and 0.5 ml of 65% polyethylene glycol (Sigma Chemical Co., St. Louis, MO) in distilled water containing 2 mM $CaCl_2$ at pH 7.4 was added and vortexed. The tubes were centrifuged at 20 000 × *g* for 30 min at 4°C. After removal of the supernatant, 0.2 ml of 0.1 N NaOH was added to dissolve the pellet, the sample vortexed and 3.5 ml of scintillation cocktail (24 g PPO, 1.2 g POPOP in 3600 ml of toluene and 400 ml Biosolv® BBS-3) were added. Biosolv, PPO, POPOP were purchased from Beckman Co. The samples were counted in a Beckman LS 330 liquid scintillation spectrometer with an efficiency for $^3$H of 40%. Non-specific binding was always less than 5%. Appropriate blank and known standards were run in each assay. The values for media alone never exceeded 2 pg/tube in any of the three assays.

*Statistical analysis*

The paired Student's *t*-test was used to compare differences between control tissues and segments of the same tissues after treatments. A *P* value of 0.05 or less was considered significant.

## Results

*Time course of gap junction development*

Electron micrographs of myometrial gap junctions at high and low magnification are shown in Figs. 1 and 2. Figure 3 shows the gap junction area in tissues from 8 women before and following incubation for up to 48 h. All tissues contained very few gap junctions before incubation. Tissues from 3 women (MC, ET, and RD) progressively developed more junctions and contained very high values after 48 h. There was little gap junction development in tissues from 5 women (VW, KB, MB, LO and VS) over the 48-h incubation period. Tissue from one postdated woman (41 weeks, VS) contained the lowest area of junctions after 48 h in vitro.

*Effects of indomethacin*

The gap junction area, in segments of the same tissues used in the control experiments (Fig. 3) but treated with indomethacin (20 μg/ml), at various times of incubation is shown in Figure 4. All tissues, except one (ET), contained gap junction areas above 0.2% after 48 h incubation.

Comparison of the gap junction areas in

**Figs. 1,2.** Electron micrographs of myometrial tissues after 48 h incubation showing gap junctions between muscle cells at intermediate (Fig. 1, ×64,000) and high magnification (Fig. 2, ×118,000).

control and indomethacin-treated (20 μg/ml) tissues at 48 h is shown in Fig. 5. There was no significant difference ($P > 0.05$) between the mean control junction area values (0.33 + 0.34% S.E.M.) and the mean values from all the indomethacin-treated tissues (0.45 + 0.21%). However, indomethacin resulted in a significant increase ($P < 0.05$) in all 5 tissues from women (KB, VS, MB, VW, LD) in which the control tissues developed very few junctions over 48 h (i.e. below 0.2%). Indomethacin reduced, but not to significant levels, the gap junction area in 3 tissues from the 3 women (MC, RD, ET) where the control tissues had many junctions at 48 h.

*Prostaglandins in the media*

The levels of prostaglandins E(PGE), F(PGF) and 13,14-dihydro-15-keto-PGF (PGF metabolite) in media collected at various times from dishes containing the control and indomethacin (20 μg/ml) treated tissues is shown in Figs. 6, 7 and 8. The amounts (normalized to g of tissue wet weight) of PGE, PGF and PGF metabolite in the media surrounding control tissues increased progressively with time. At 48 h there was about 3 times as much PGE in the media as PGF. Also, at 48 h there was approximately 4 times as much PGF as PGF metabolite. The levels of PGE and PGF were

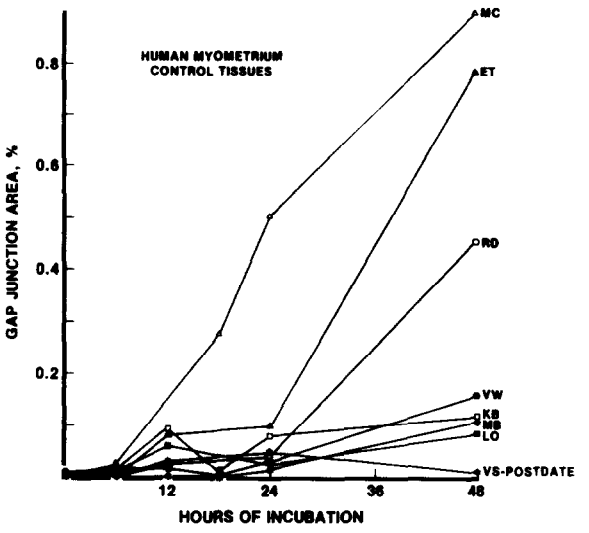


Fig. 3. Gap junction area, as % of plasma membrane area, in control tissues from 8 women at 0 h and at various times up to 48 h incubation.

significantly ($P < 0.05$) reduced at all times by indomethacin. However, indomethacin did not prevent the initial rise in PGF metabolite.

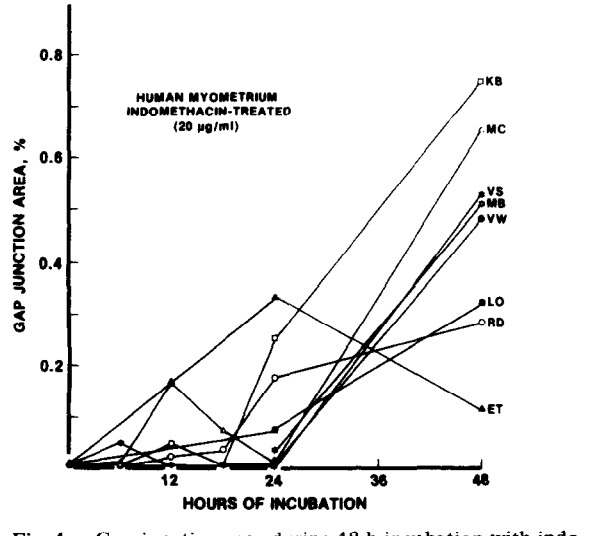


Fig. 4. Gap junction area during 48 h incubation with indomethacin (20 µg/ml) from tissues of the same women shown in Fig. 3.

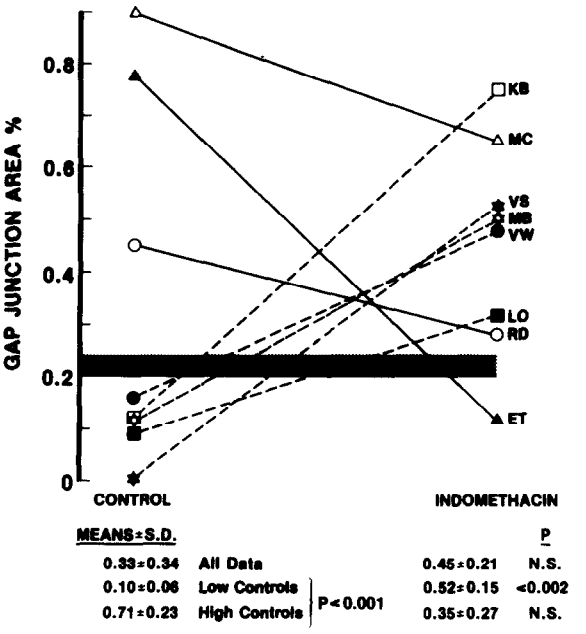


Fig. 5. Comparison of the gap junction area after 48 h incubation in the control and indomethacin-treated tissues. Note that there were no significant differences ($P < 0.05$) between control and indomethacin gap junction values when all data was considered. However, tissues from 5 women (KB, VS, MB, VW, LD) with low gap junction areas (i.e. below 0.2%), contained significantly ($P < 0.002$) higher gap junction areas after treatment with indomethacin. Tissues from 3 women (MC, RD, ET) with high control values contained less after incubation with indomethacin but the mean values were not significantly different ($P > 0.05$).

## Discussion

This study shows that myometrial gap junctions develop slowly in some of the tissues in vitro. Tissues removed from women prior to labor contained very few gap junctions but segments of some of the same tissues contained more after incubation. These results are similar to those seen in animal tissues studied in vitro [7,8]. However, it is obvious that tissues from other women had less capacity to form gap junctions. These results can perhaps be explained on the basis that the development and presence of gap junctions in vitro or in vivo is the result of maturational events in prepara-

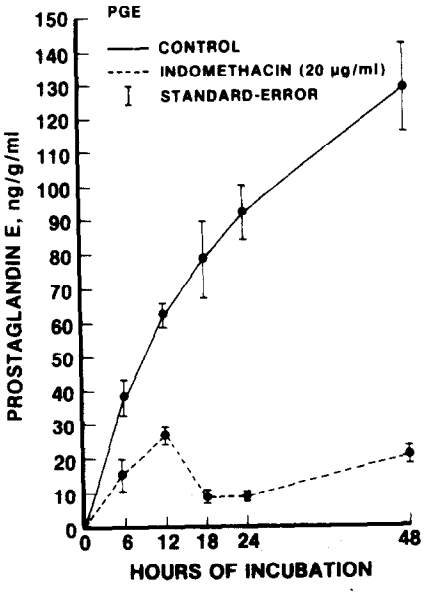


Fig. 6. Levels of prostaglandin E (ng PGE/g myometrial per ml of MEM) in the incubation media bathing tissues in the absence (control) and presence of indomethacin (20 µg/ml). Points are mean values ± S.E.M. from 4 separate tissues.

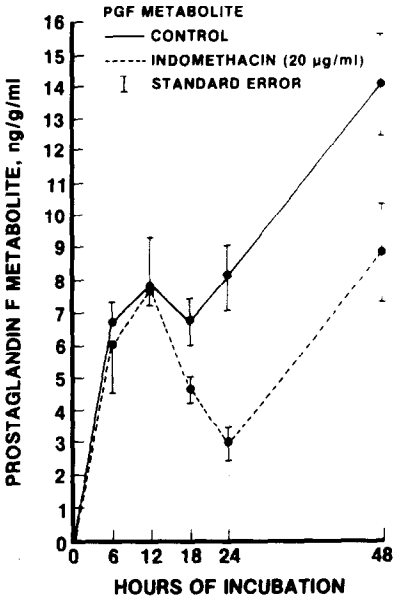


Fig. 8. Levels of 13,14-dihydro-15-keto prostaglandin F (PGF metabolite) in the bathing media from control and indomethacin-treated tissues. Data as shown in Fig. 6.

tion for labor and that the tissues used in this study were from women at different stages of that maturational process.

It should be noted that any change in gap junctions could be the result of changes in their synthesis and/or degradation. It is obvious from the present study that tissues which develop large numbers of junctions in vitro, either spontaneously or after indomethacin treatment, do so over an extended period (i.e. 24–48 h, see Figs. 3 and 4). This time period may be equivalent to the time required in vivo for the presence of sufficient junctions for labor to progress. We assume that the presence of the junctions is the result of increased synthesis as is thought to be the case for myometrial tissues from animals [10].

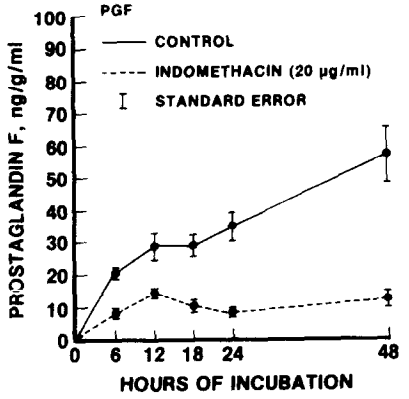


Fig. 7. Levels of prostaglandin F (PGF) in the incubation media from control and indomethacin-treated tissues. Data as shown in Fig. 6.

It is obvious that the presence of the placenta, decidua, fetal membranes and fetus are not necessary for gap junctions to form in vitro as our tissue specimens were primarily muscle. However, these tissues may modulate the presence of the junctions through the production of prostaglandins and other regulatory molecules or mediate the action of the steroid hormones [see 19].

Our results show that the tissues released PGE, PGF and PGF metabolite into the incubation media (Figs. 6–8) and that the

amounts of prostaglandins were reduced significantly by indomethacin treatment. These results are in agreement with reports by others [3,4]. Similarly our study shows that more PGE is produced than PGF which corresponds with prior observations [19]. However, the prostanoids we measured in this study may not be the ones involved in regulation of the junctions.

Previously we have found in human and animal tissues that labor and delivery occurred when approximately 0.2–0.3% of the plasma membrane of myometrial cells was occupied by gap junctions [11,17]. In this study we show that indomethacin increases gap junction area in segments of those same tissues which developed less than 0.2% of membrane area in control incubations (Fig. 5). This may mean that a product of the cyclooxygenase pathway prevents gap junction development and that a shift in the synthesis of the cyclooxygenase inhibitory product is one of the steps involved in the maturational process in preparation for labor. Our previous studies suggest that prostacyclin ($PGI_2$), a cyclooxygenase product, may inhibit the presence of myometrial gap junctions [8]. Other studies in animals also show that indomethacin and meclofenamate treatment increase gap junction area [14]. These results support the notion that $PGI_2$ prevents gap junction development. The concept that a change in the synthesis from $PGI_2$ to other prostanoids may be part of the maturational events prior to labor has been proposed [1,16]. Another possibility to explain the above results is that products of the lipogenase pathway regulate (stimulate) the presence of the junctions and that when the activity of the cycloxygenase enzyme is inhibited with indomethacin, arachidonic metabolism is shifted to the lipoxygenase pathway and production of leukotrienes.

In contrast, indomethacin consistently prevented an increase in gap junctions in tissues in which control pieces of the same tissues developed many junctions (Fig. 5). These results may indicate that more than one prostanoid is involved in either controlling the synthesis or degradation of the junctions. Another possibility is that the presence of myometrial gap junctions is controlled by a balance between production from the cyclooxygenase and lipoxygenase pathways. The differences observed in the ability of indomethacin to induce gap junctions in some tissues and prevent their appearance in others is also supported by previous studies. Indomethacin inhibits gap junctions in rat myometrial tissues in vitro (i.e. in tissues which develop many junctions in vitro [8]), and the drug stimulates gap junctions in myometrial tissues in animals treated with indomethacin plus estradiol in vivo [14,15].

Our study has important implications in the management of labor. Inhibition of prostaglandin synthesis may stimulate the presence of gap junctions in some patients as well as decrease the synthesis of products which may directly accelerate and augment contractility. Further studies are needed to define fully the mechanisms involved in the control of myometrial gap junctions and their involvement in labor.

## Acknowledgments

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