Changes in expression of $P2X_1$ receptors and connexin 43 in the rat myometrium during pregnancy

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Objective: To investigate the expression of $P2X_1$ receptors and connexin 43 in gap junctions between smooth muscle cells. Contraction mediated by P2X receptors is known to occur in the bladder and male reproductive tract, and cell–cell coupling of smooth muscle via gap junctions is essential for synchronized rhythmic activity of these tissues.
Design: We selected for this study rat myometrial smooth muscle during pregnancy and at postpartum day l.
Setting: University medical school.
Animal(s): Laboratory rats.
Intervention(s): Rats were mated and became pregnant.
Main Outcome Measure(s): Immunostaining and fluorescence and confocal microscopy.
Result(s): The level of $P2X_1$ receptor expression remained low throughout pregnancy (days 4 to 20) but was greatly up-regulated at day 22 (postpartum day 1). Connexin 43 expression showed a pattern of up-regulation, with progression through pregnancy and peaking near labor, but exhibited a rapid down-regulation after parturition.
Conclusion(s): The functional significance of the changes in connexin 43 and $P2X_1$ receptor expression that have been observed is discussed in relation to triggering and modulation of uterine contractility during and after pregnancy. (Fertil Steril® 2007;88(Suppl 2):1174–9. ©2007 by American Society for Reproductive Medicine.)

Key Words: $P2X_1$ receptor, connexin 43, myometrium, rat, confocal microscopy, immunofluorescence

A recent article has shown that $P2X_1$ receptors are closely associated with connexin 43 (Cx43) gap junctions in the human ventricular myocardium and may be involved in gap-junction formation (1). Gap junctions are intercellular channels, composed of a pair of connexon proteins, which are comprised of connexins in a hexameric organization (2). Twenty-one human connexin genes have been identified thus far (3). Gap junctions are found in abundance between smooth muscle cells (4, 5). They appear to play roles in coordinating cellular signals during growth and development (6). Adenosine 5′-triphosphate (ATP) acts on P2 receptors, which consist of a family of ligand-gated ion channels (P2X receptors) and a family of G protein–coupled receptors (P2Y receptors) (7). Adenosine 5′-triphosphate is released as a co-transmitter with noradrenaline from sympathetic nerves (8, 9), and extracellular ATP is a signaling molecule in many nonneuronal as well as neuronal cells (10).

There are reports that ATP is involved with connexins in gap-junction activity. For instance, it is suggested that in murine macrophages, Cx43 forms so-called half-gap junctions in response to ATP (11), and ATP is able to pass 300 times more efficiently through channels that are composed of Cx43 than are AMP or ADP in C6 glioma cells transfected with connexin particles (12). Intercellular calcium-wave propagation in many cell types involves interplay between connexins and P2 receptor-mediated processes. For example, interplay between P2 receptors and gap junctions in intercellular communication has been proposed in Hensen's cells (13) and in airway epithelia (14). In astrocytes, it has been demonstrated that down-modulation of gap-junctional communication, by using cytokines or gene targeting, results in altered expression of P2 receptors of the $P2Y_1$ receptor subtype (15).

The myometrium has two distinct layers of smooth muscle, an outer longitudinal layer and an inner circular layer. Uterine contractility depends on the propagation of action potentials between myometrial cells, and gap junctions play a key role in regulating myometrial activity. Coordination of uterine contractions is essential during delivery, and studies in different mammalian species have shown that labor is preceded by increases in myometrial gap-junction communication (16–18). In this context, we investigated the expression of $P2X_1$ receptors and Cx43 gap junctions between rat myometrial smooth muscle cells at three stages of pregnancy and at postpartum day 1.

MATERIALS AND METHODS

Animals

The use of animals followed principles of good laboratory animal care and experimentation, in compliance with United Kingdom Home Office regulations covering so-called Schedule One Procedures, as well as in accordance with the 1986 United Kingdom Animals (Scientific Procedures) Act and with ethical approval. Experiments were performed by using

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female sexually mature Sprague-Dawley rats, which have a gestation period of 21 days. Studies were conducted at early pregnancy (day 4), mid pregnancy (day 14), and late pregnancy (day 20) and at postpartum day 1. Four animals from each stage were used.

Animals were killed by asphyxiation with CO_2, followed by cervical dislocation. The middle section of each uterine horn was carefully removed and placed in Hanks' balanced salt solution (VWR International, Poole, UK). The fetuses were removed from the intact uterine horns and killed by using a Schedule One procedure. Gestation day 1 was determined by the presence of a vaginal plug.

The tissues were embedded longitudinally in Tissue Tek OCT compound (Sakura, Zoederwoude, the Netherlands) and frozen in precooled (in liquid nitrogen) isopentane. Tissues were sectioned at 12 μm with a Reichert Jung CMI800 cryostat and were collected on gelatin-coated slides. The slides were air-dried at room temperature and were stored at -20°C until needed.

Immunolabeling With $P2X_1$ Receptor and Cx43 Antibodies

The development and specificity of the anti-$P2X_1$ receptor polyclonal antibody (Roche, Palo Alto, CA) and the GAP 15 rabbit polyclonal Cx43 antibody (to short peptide sequences of Cx43) have been reported elsewhere (19–22). Air-dried sections were fixed in 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 minutes. They were washed in phosphate-buffered saline (PBS) solution for 15 minutes and then were incubated in 10% normal horse serum for 20 minutes to block nonspecific binding sites. This step was repeated by using 10% normal goat serum to block nonspecific binding sites for Cx43.

The slides were incubated overnight at room temperature in 2.5 μg/mL of primary $P2X_1$ receptor antibody diluted with 10% normal horse serum–PBS–merthiolate. Further washes were performed by using PBS containing 0.05% merthiolate (Sigma Chemical Co., Poole, UK). After washing, the slides were further incubated for 1 hour in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in 1% normal horse serum–PBS–merthiolate. After another 15-minute wash, $P2X_1$ receptor expression was visualized by using Texas Red streptavidin (Sigma), diluted 1:100 with 10% normal horse serum for 1 hour.

Consecutive sections of comparable areas of the myometrium to those stained with the $P2X_1$ receptor antibody were incubated with the Cx43 antibody, diluted 1:50 with 10% normal goat serum overnight at room temperature. Sections were then washed for 15 minutes, and Cx43 expression was visualized by incubating with streptavidin–fluorescein isothiocyanate labeling (Jackson) diluted 1:100 with 10% normal goat serum for 1 hour. Nuclei were visualized by the addition of 4′6, diamidino-2-phenylindole (1:3,500; Sigma) with the secondary antibody. A final wash was performed in PBS, and then the sections were mounted immediately in Citifluor (Citifluor, London, United Kingdom).

Preabsorption of the $P2X_1$ receptor antibody with an excess of the synthetic peptide eliminated immunoreactivity (23).

Photography and Image Analysis

The $P2X_1$ receptor and Cx43 plaques were viewed from the longitudinal myometrial layers and imaged blind by using confocal microscopy. Images of immunofluorescence labeling were taken with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) that was attached to a Leica SP2 confocal microscope (Leica) immediately after immunostaining. Optical images were stored digitally by using Adobe Photoshop 5 (Adobe, San Jose, CA).

Five randomly selected fields were chosen from each slide (and 10 sections per animal) and optically sectioned in three steps, the sum of which were used. These sections were scanned under identical parameters for pinhole size, objective, filters, and laser power. Scan averaging (average of 3) was used to reduce background noise. Procedures were followed to ensure that the full dynamic range available (i.e., 0–255 pixel intensity) was used. The tissue section was scanned, and optical sections were taken at successive depths within the myometrium from its apex down to the base. Thus the Z-series sections could then be reconstructed to obtain a topographical three-dimensional image and to enable the exact distribution of the staining at each level to be viewed. All scanning was performed at high magnification ($\times$63).

The levels of Cx43 and $P2X_1$ receptor expression were analyzed on digital images at each stage. Standard magnification ($\times$63), gain, and pinhole settings of the confocal microscope were used to achieve the best possible signal-to-noise ratio. Digital single-channel images were transformed into 8-bit black-and-white format and were analyzed by using NIH Image software (http://rsb.info.nih.gov/nih-image). At standard threshold settings, which ensured that only specific signals were considered and the background was excluded, the area fractions of Cx43 and $P2X_1$ receptor immunofluorescent plaques were measured on each image. The means of percentages in each group were summarized in graphs.

Statistical Analysis

The number of particles of Cx43 and $P2X_1$ receptor immunofluorescence for each field of view was counted. The data from each field of view from all the slides per animal were pooled, and then the mean was calculated to give the mean total number of particles per view (158.73 μm^2) $\pm$ SEM, for each stage of pregnancy studied as well as for 1 day postpartum. These were compared statistically by using a one-way analysis of variance, followed by a Bonferroni post hoc test. Although each group of data was compared with all the other groups, significance is indicated on the histograms only when a group

is significantly different from the preceding group. A probability of $P<.05$ was considered to be significant.

RESULTS

During the stages of pregnancy examined, Cx43 expression was detected, although the level of expression varied (Fig. 1a–d). Labeling of Cx43 was easily distinguished in the longitudinal myometrial layer from background or contaminants by virtue of its size and cellular location. The Cx43 plaques always appeared as very bright, fluorescing punctata of size <2 μm, located at cell–cell boundaries.

The number of Cx43 particles increased as pregnancy progressed (Fig. 2a). The number of Cx43 particles had increased significantly ($P<.001$), by 1718% by day 14 (Fig. 1b) and by 2773% by day 20 (Fig. 1c), compared with day 4. At postpartum day 1 (Fig. 1d), the level of Cx43 had significantly ($P<.001$) decreased, to a level similar to that of day 4. At postpartum day 1, the staining of Cx43 may be associated only with a subpopulation of macrophage-like cells.

At days 4, 14, and 20, there was a low level of $P2X_1$ receptor immunofluorescence (Fig. 1e–h). There was no significant difference in the number of $P2X_1$ receptor particles from days 4 and 14, but the number of particles decreased significantly ($P<.01$), to 36.6% of that at day 4. At postpartum day 1, the number of $P2X_1$ receptor particles increased significantly ($P<.001$; Fig. 2b) compared with days 4, 14, and 20, being 219% greater than the number on day 4 and visible as bright fluorescing punctata.

DISCUSSION

Connexin proteins form gap junctions to create low-resistance pathways for the propagation of low-threshold stimuli throughout the smooth muscle. This study aimed to examine the expression of Cx43 and $P2X_1$ receptors in the rat myometrium, during pregnancy and immediately postpartum, when immense plastic changes occur to accommodate the growing fetus and to prepare for parturition. The myometrial smooth muscle cells undergo hyperplasia and hypertrophy (24), and the density of innervation of the uterus decreases as pregnancy progresses (25). After parturition, the uterus regains its nonpregnant state quickly, so death of uterine cells may take place. This study suggests that myometrial smooth muscle contractility is modulated by the differential yet contrasting distribution of $P2X_1$ receptors, compared with Cx43.

Uterine gap junctions have been documented elsewhere (26, 27) and have been shown to increase during pregnancy (28). This study found that with the progression of pregnancy, the levels of Cx43 increased, with a striking up-regulation at day 20, the day before parturition. This up-regulation of Cx43 and formation of functional gap junctions during the period immediately surrounding parturition has been demonstrated elsewhere (26, 29–31) and may reflect the control that a fetus exerts upon pregnancy and the lack of maternal control. If the fetus is modulating pregnancy, it will do so by releasing

FIGURE 1

(**a–d**) Labeling of rat myometrial smooth muscle cells for connexin 43 (*green*) and counterstaining with 4′6, diamidino-2-phenylindole to visualize the nuclei (*blue*) during pregnancy at different stages: (**a**) day 4, (**b**) day 14, (**c**) day 20, and (**d**) day 1 postpartum. Note the increase in connexin 43 staining at day 14 and day 20. At day 1 postpartum, connexin 43 staining was only observed within a subpopulation of macrophage-like cells (*arrows*). Bars = 20 μm. (**e–h**) Immunolabeling of rat myometrial smooth muscle cells for $P2X_1$ receptors (*red*) during pregnancy at different stages: (**a**) day 4, (**b**) day 14, (**c**) day 20, and (**d**) day 1 postpartum. Note the increase in expression of $P2X_1$ receptors in (**d**). Bars = 20 μm.

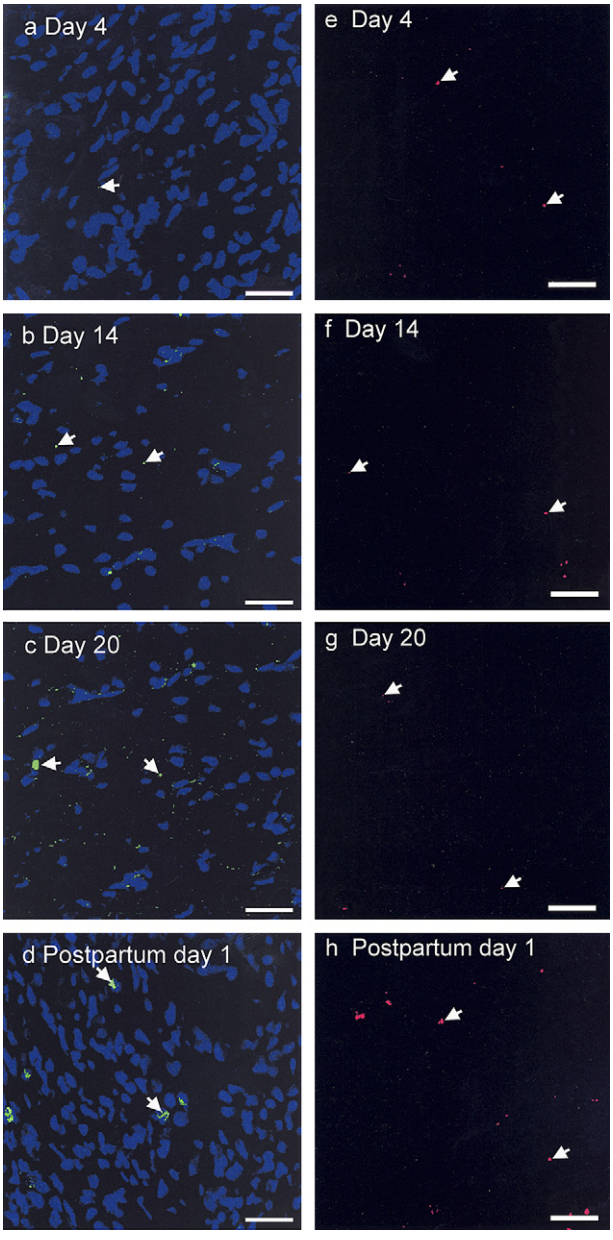

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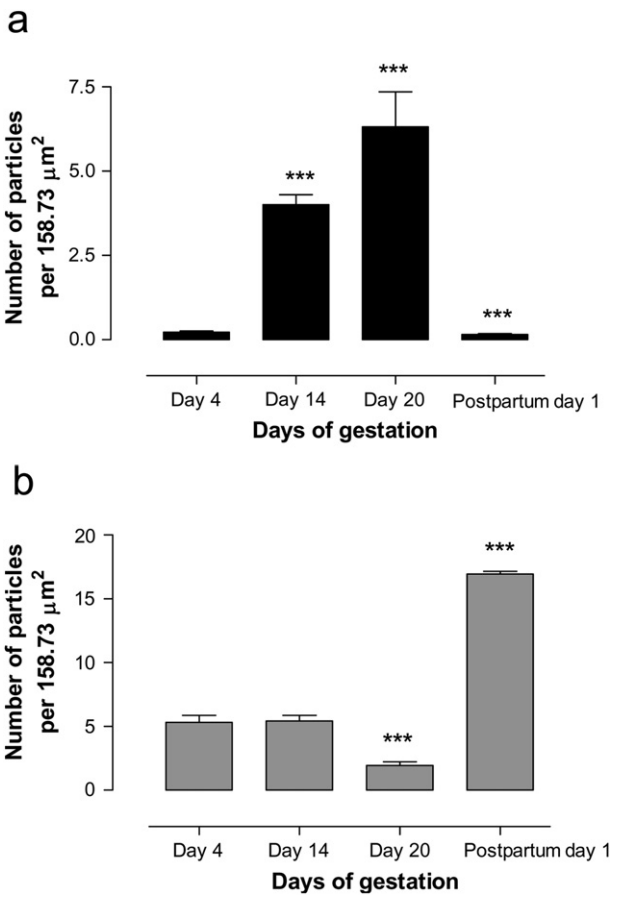
FIGURE 2

Histograms showing the results of the quantification of connexin 43 (**a**) and $P2X_1$ receptors (**b**) in rat myometrial smooth muscle cells during pregnancy and 1 day postpartum. *Bars* represent the mean number of particles per 158.73 μm^2 ($\pm$ SEM). Statistical significance is indicated where a data set is significantly different from the preceding data set: ***$P<.001$.



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hormones (such as hCG and prostaglandins), so it is critical that the whole uterus act as a syncytia and be responsive to low levels of such hormones (32). For this to be feasible, better electrical and metabolic coupling of the uterine cells is essential.

There is an increased demand for better cell-to-cell communication at the later stages of pregnancy, to enable the uterine smooth muscle as a whole to act in concert to bring about effective propulsion of the fetus at parturition (33). Our findings (of an increase in Cx43 during late pregnancy) are consistent with this. Connexin 43 was evenly distributed throughout the myometrial tissue at day 20, and the plaque sizes appeared larger, suggesting a role in the preparation for coordinated contraction at parturition. This may also reflect a compensatory mechanism to maintain as constant the number of gap junctions per myometrial smooth muscle cell (hypertrophied) (34).

At 1 day postpartum, significant down-regulation of Cx43 was detected, and the distribution was limited to a subpopulation of cells. Because apoptosis is a feature in this stage (35), it is likely that cells associated with programmed cell death also may be present, including macrophages and eosinophils. The presence of Cx43 in macrophages is well documented (11, 36). It is possible that the Cx43 immunoreactivity at 1 day postpartum may be associated with macrophages, which are known to increase in number in the myometrium at parturition and are thought to have a role in uterine contractility (37). However, further studies need to be performed to confirm this.

Weak $P2X_1$ receptor immunostaining of myometrium was seen during pregnancy; this decreased before parturition and significantly increased 1 day after parturition. This staining pattern contrasts with that of Cx43. Although the $P2X_1$ receptor staining was not colocalized with Cx43, in contrast to the human heart, in which colocalization has been shown (1), the increase in $P2X_1$ receptor expression after parturition may by involved in inhibiting Cx43 expression, thereby reducing the number of gap junctions and avoiding coordinated smooth muscle contractions. With finer maternal control of the uterus after parturition, remodeling, resorption, and reversal of the hypertrophy is possible. This up-regulation also corresponds to the period of uterine involution (i.e., the lumen is folded), and $P2X_1$ receptors may have a role in the stronger contraction occurring at this time.

Very weak $P2X_1$ receptor immunoreactivity of the nonpregnant rat uterus has been described (23), and $P2X_1$ receptors have been identified in the uterine epithelial cells in nonpregnant rats and during early pregnancy (38, 39). Adenosine 5′-triphosphate causes direct contraction of rodent myometrial smooth muscle cells in the nonpregnant (40–43) and pregnant myometrium (44, 45), probably acting via $P2X_1$ receptors. In the human pregnant uterus, contractions caused by $P2X_1$ receptor stimulation increased with the progression of pregnancy (46), consistent with this study, in which expression of $P2X_1$ receptors increased during pregnancy.

The sympathetic innervation of the uterus is widespread, and these nerves may provide the main source of extracellular ATP within the uterus, ATP that is being released by exocytosis (47, 48). Adenosine 5′-triphosphate also may be released from nonneuronal sources. There is strong evidence for physiological ATP release from various cell types, including endothelial and epithelial cells and smooth muscle, triggered by mechanical distortion of cells (49) as well as from damaged cells. In the case of the myometrium, which is subjected to increasing mechanical stress as the fetus grows, followed by the trauma of parturition, smooth muscle and/or epithelial cells could release ATP.

With hypertrophy of myometrial cells, many new capillaries are formed (50). Subsequent to the increase in demand for blood supply during pregnancy, the concomitant increase in

vascularization and extracellular ATP may up-regulate and activate more P2X receptors. Receptors for $P2X_1$ have been found in many vasculatures (10). It is possible that this cascade of events assists the uterus in accommodating the fetus and maintaining muscular and vascular tone (51).

Uterine quiescence is vital until parturition to ensure the survival and effective development of the fetus and to avoid abortion. Many factors play a role in the control of smooth muscle contraction. The low levels of Cx43 at earlier stages of pregnancy may be associated with a lower level of coordinated contractile activity of the muscle cells. Sladek et al. (52) postulated that endogenous nitric oxide suppresses the expression of Cx43 in the myometrium. At parturition, with increased levels of gap junctions in the hypertrophied myometrial smooth muscle, the electrical coupling is reported to be more efficient, and this may modulate the contractile activity.

In conclusion, this study has shown that the expression of Cx43 is up-regulated in the later stages of pregnancy and peaks near parturition. This may echo the significant role that gap junctions play in the synchronized and coordinated uterine contractions at parturition. The presence of $P2X_1$ receptors in the myometrium supports an important role for ATP as an extracellular messenger that is involved in purinergic signaling in the myometrium. However, the pattern of expression of the $P2X_1$ receptors differs from that of Cx43, with a peak expression at 1 day after parturition. Because colocalization of $P2X_1$ receptors and Cx43 was not seen, they may play different roles, and it is suggested that the high levels of ATP that are released from uterine cells during childbirth may act on $P2X_1$ receptors in gap junctions to inhibit expression of Cx43 and thereby reduce gap junctions and coordinated contraction.

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