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Pregnancy-associated changes in responsiveness of the porcine myometrium to bioactive substances

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Abstract

To determine the pregnancy-associated changes in the porcine uterine contractility, the spontaneous contraction and the mechanical responses to bioactive substances of uteri in nonpregnant proestrus and pregnant pigs (25–60 days of gestation) were compared in vitro. Longitudinal muscle (LM) and circular muscle (CM) of the uterus exhibited spontaneous contraction, but the frequency in pregnant pigs was lower than that in the nonpregnant pigs. The duration and force of spontaneous contraction in the pregnant pigs were long and large compared with both in the nonpregnant pigs. L-Nitroarginine methylester (L-NAME) and 2*a*-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2*a*,3,4,5-tetrahydro-benzo[*cd*]indol-2(1*H*)-one (DR4004) did not change the spontaneous contraction in the uteri of nonpregnant pigs but increased its amplitude in the uteri of pregnant pigs. Isoprenaline inhibited the uterine spontaneous contraction of the nonpregnant and pregnant pigs, and the inhibition was stronger in the pregnant than in the nonpregnant pigs. 5-Hydroxytryptamine also caused inhibition of spontaneous contraction in the uteri of nonpregnant pigs (CM>LM). In the pregnant pigs, sensitivity to 5-hydroxytryptamine increased in a muscle layer-dependent manner (LM>CM) and difference in the responsiveness between LM and CM decreased. Acetylcholine contracted the uterine LM and CM strips of the pregnant and nonpregnant pigs. The responsiveness of CM increased slightly during pregnancy, but that of the LM did not change. 5-Bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine (UK14304) caused contraction of only LM in the uteri of nonpregnant pigs, but contracted both LM and CM strips in the pregnant pigs. Oxytocin and prostaglandin $F_{2\alpha}$ also contracted the uteri of nonpregnant pigs (LM>CM). Pregnancy increased the contraction of both agents in the LM and CM, but the increment was marked in the CM. The contractile forces induced by all stimulants were increased (by 1.7- to 2.5-fold) in the LM and CM of pregnant pigs. In conclusion, (1) low frequency, slow kinetics and large force of spontaneous contraction are characteristics of the pregnant porcine uteri, and nitric oxide and 5-hydroxytryptamine are supposed to be partially involved in the regulation of spontaneous contraction, and (2) responses to both contractile and inhibitory agents are increased in the pregnant pigs. Increment of the responsiveness is conspicuous in the muscle layer that is less sensitive to each agonist in the uteri of nonpregnant pigs. According to the pregnancy-associated changes, muscle layer-related differences of responsiveness to bioactive substances in the nonpregnant pigs tend to decrease in the pregnant pigs.

Keywords: Porcine uterus; Pregnancy; Spontaneous contraction; Acetylcholine; Oxytocin; 5-Hydroxytryptamine; Nitric oxide

1. Introduction

It is generally accepted that uterine activity is regulated by complex and mutual interactions among sex steroid hormones, myometrial contractility, autonomic innervation [excitatory cholinergic, excitatory (α-adrenoceptor) and inhibitory (β-adrenoceptor) adrenergic and peptidergic nerves] and some autacoids (histamine, 5-hydroxytryptamine, prostaglandins and nitric oxide). In the previous studies, we have used uteri of the nonpregnant proestrus pigs due to the easy separability of longitudinal muscle (LM) and circular muscle (CM), and have reported muscle layer-related differences in autonomic innervation and in the responsiveness to some bioactive substances. The porcine cornual LM is innervated by adrenergic nerves, but the CM is innervated by cholinergic nerves. Although the LM and CM responded to bioactive substances, the responsiveness to acetylcholine (muscarinic M_3 receptor), noradrenaline (α_2-adrenoceptor), histamine (histamine H_1 receptor), endothelin (endothelin ET_A receptor) and oxytocin (oxytocin receptor) was always higher in the LM than in the CM. Similarly, the sensitivity to relaxant substances, such as 5-

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hydroxytryptamine (5-HT_7 receptor) and isoprenaline (β_2-adrenoceptor), was also different in the LM and CM. 5-HT_7 receptor and β_2-adrenoceptor shared in inhibition of the porcine uterine contractility in a muscle layer-dependent manner (LM: β_2-adrenoceptors; CM: 5-HT_7 receptors) (Taneike et al., 1991, 1994, 1995; Kitazawa et al., 1997, 1998, 2000, 2001a,b; Isaka et al., 2000). The muscle layer-specific responsiveness is a unique characteristic in the uteri of nonpregnant pigs, due to the heterogeneous distribution of respective receptors, and might reflect the inherent properties derived from fetal-stage differentiation of the two muscle layers during organogenesis.

During the estrous cycle (nonpregnancy), pregnancy and parturition (delivery), the uterus undergoes a rapid transformation from an active state (estrous cycle) to a state of quiescence (pregnancy) and finally to forceful contraction characteristics of labor. Mechanisms underlying gestation-related changes in uterine activity include alterations in hormonal, metabolic, and neural inputs to the uterus as well as changes in the responsiveness of the myometrium to bioactive substances through alteration in receptors and coupled signal transduction mechanisms. In the rat uterus, density of β-adrenoceptors, adenylate cyclase activity and β-adrenoceptor-mediated relaxation increased during pregnancy, but they were decreased abruptly to nonpregnant levels on the last day of pregnancy and may be contributing mechanisms for the initiation of labor (Cohen-Tannoudji et al., 1991; Engstrom et al., 1997; Lindeman et al., 2000). Recent studies have indicated pregnancy- and labor-related changes in the mechanical responses to L-arginine (nitric oxide synthase substrate). L-Arginine-induced inhibition of uterine contractility was conspicuous in non-delivering pregnant rats but was dramatically decreased at the delivery and at the postpartum days. These studies indicate that nitric oxide may contribute to the quiescence of the uterus during pregnancy (Yallampalli et al., 1994; Buhimschi et al., 1995). A pregnancy-related increase in sensitivity to oxytocin occurred in several animal species (rat, human, guinea-pig and rabbit) and has been shown to be caused by an increase in the density of oxytocin receptors (Fuchs et al., 1982, 1984). In addition, marked increases in the force of contractile responses to high-K^+, prostaglandin $F_{2\alpha}$ and carbachol at the end of pregnancy have been reported (Kim et al., 1998). Although many studies on uteri of small experimental animals (mostly rats) have indicated that there are a pregnancy-related increase in the sensitivity to inhibitory agents and a delivery-related increase in the sensitivity to contractile agents, there have been very few studies on spontaneous mechanical activities and drug sensitivities of the uteri of pregnant domestic animals such as pigs.

In the present study, we obtained uteri of both nonpregnant (proestrus) and pregnant pigs from a local abattoir and prepared LM and CM strips from the cornual region. The muscle preparations were suspended in an organ bath, and characteristics of the spontaneous contractions and the responsiveness to contractile [acetylcholine, 5-bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine (UK14304), prostaglandin $F_{2\alpha}$ and oxytocin] and relaxatory agents (5-hydroxytryptamine and isoprenaline) were compared to determine the pregnancy-associated changes in the porcine uteri.

2. Materials and methods

2.1. Tissue preparations

Fresh uteri were obtained from 30 adult crossbred nonpregnant (6–8 months old) and 16 pregnant pigs at a local abattoir. Based on gross examination of the follicle size (<2 mm) and appearance of the corpora lutea, only the uteri of nonpregnant pigs judged to be in proestrus were used (McDonald, 1975). In the case of pregnant pigs, gestation stage was estimated by length of the fetus (from head to tail length) based on the following equation: $Y = 0.3057X - 6.2899$, where Y is the length of the fetus (cm) and X is the day of gestation (Kawano and Sakakibara, 1998). In this study, we used the uteri of pregnant pigs with fetuses of 20–120 mm in length, corresponding to the first half stage of gestation (25–60 gestation days, general gestation period of pigs being 114 days). Experiments described below were conducted on the day of slaughter (within 2–3 h). LM and CM strips of the porcine uterus were prepared from the antimesometrial coat of the adtubal region (10 cm distal from the apex) in either left or right cornua by surgical procedures described previously (Kitazawa et al., 2000, 2001b). In brief, after removal of the endometrium, muscle strips parallel to the direction of the CM and LM fibers were isolated. Then the unwanted muscle was removed from each muscle layer by meticulously cutting with fine scissors under a binocular microscope. One or two muscle preparations were obtained from the uterus of each nonpregnant and pregnant pig, and used in the following contraction study.

2.2. Contraction study

Each LM and CM strip (1 mm width, 10 mm length, 0.5 mm thick, 15–20 mg wet weight) was suspended vertically in an organ bath (20 ml) containing 37 °C Krebs solution (mM) (NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11.5) bubbled with 95% O_2 + 5% CO_2 (pH = 7.4). A force-displacement transducer (SB-1T, Nihon Kohden) equipped with a pen-writing recorder (Recticorder, Nihon Kohden) was used to measure the mechanical activity of the myometrial preparations. The muscle strips were loaded at 3.9 mN as an initial tension (this tension was adequate to produce the regular spontaneous contraction and reproducible responses to agents) and allowed to equilibrate for 90 min. During equilibration, all LM and CM layers of the uteri from nonpregnant pigs and the CM layers of the uteri from pregnant pigs showed the spontaneous contraction but some uterine LM strips of pregnant pigs did not contract sponta-

neously (see below). To compare the profiles of spontaneous contraction in the uteri of both pregnant and nonpregnant pigs, the effects of tetrodotoxin (1 μM), verapamil (10 μM) and incubation in Ca^{2+}-free Krebs solution were examined.

In the present experiments, the responsiveness of contractile (acetylcholine, UK14304, oxytocin and prostaglandin $F_{2\alpha}$) and relaxatory agents (5-hydroxytryptamine and isoprenaline) was compared between nonpregnant and pregnant uteri. Because 5-hydroxytryptamine and isoprenaline inhibited the spontaneous contraction (Kitazawa et al., 1998, 2000, 2001b), the effects of both agents were needed to investigate in the myometrial strips exhibiting spontaneous contraction. Therefore, after 90 min equilibration, if the uterine smooth muscle strips showed spontaneous contractile activity, the responses to isoprenaline and 5-hydroxytryptamine were examined first, and then the effects of four contractile agents were analyzed in the same preparation. However, if myometrial strips did not contract spontaneously (in case of LM strips from the uteri of pregnant pigs), the effects of the contractile agents were tested and then second (another) LM strip from the same pregnant uterus was prepared. If the second uterine preparation exhibited the spontaneous contraction, the effects of relaxatory agents were analyzed, but it did not show the spontaneous contraction; acquisition of the data concerning the inhibitory agents was finally given up. In the first 16 LM strips from the uteri of 16 pregnant pigs, 7 strips did not show the spontaneous contraction even after 90 min equilibration. In the 7 second LM strips from 7 pregnant pigs, the first preparation of which failed to contract spontaneously, 4 strips did not show the spontaneous contraction after all. Totally, 23 LM strips of 16 pregnant porcine uteri were used in the present experiments (9 pigs: 1 strip isolated from each pig; 7 pigs: 2 strips isolated from each pig), and 12 strips contracted spontaneously but 11 strips failed to show spontaneous contraction. After obtaining the reproducible spontaneous contraction, 5-hydroxytryptamine or isoprenaline was applied cumulatively to an organ bath at 5-min intervals (inhibitory responses of both agents reached plateau within 3–5 min), and the amplitude of the maximally inhibited spontaneous contraction during each cycle was expressed as a percentage of the contraction in the absence of agents. During 40 min wait time after washing out of agents, inhibition of spontaneous contraction recovered completely and re-application of 5-hydroxytryptamine or isoprenaline in the same preparation caused the reproducible responses. The order of the application of two agents was sometimes varied in the different preparations. The IC_{50} value (concentration of agents that caused 50% of maximum inhibition) and maximum inhibition were determined by least-squares nonlinear regression analysis of the concentration–response curves. To compare the responsiveness of contractile agents, first, each muscle strip was subjected to stimulation with 50 mM high-K^+ (2 M KCl was directly applied in the bath) every 15 min until reproducible contraction was established, and then the concentration–contraction relationships of the agents (acetylcholine and UK14304) were determined by cumulative application with 1- to 2-min intervals. After washing out of drugs, 40 min equilibration was enough to obtain the reproducible contraction. At the end of each experiment, wet weight of myometrial strips was measured and the contraction was expressed as a force of contraction (N/g tissue wet weight) and concentration–response curves were constructed. The EC_{50} values and maximum contraction force were estimated from these concentration–response curves by least-squares nonlinear regression analysis. Contractile responses to prostaglandin $F_{2\alpha}$ and oxytocin in myometrium from the uteri of pregnant and nonpregnant pigs were also compared. Tenfold increasing concentrations of prostaglandin $F_{2\alpha}$ (100 nM, 1 and 10 μM) and oxytocin (0.1, 1, 10 and 100 nM) were added to the organ bath for 5 min at 30-min intervals, and the induced mechanical responses were evaluated by the elevation of baseline tonus and expressed as N/g tissue wet weight. The order in application of contractile substances was not fixed and varied randomly in each preparation.

2.3. Chemicals and drugs

The following chemicals and drugs were used in these experiments: acetylcholine hydrochloride (Wako), 5-bromo-*N*-(2-imidazolin-2-yl)-6-quinoxalinamine (UK14304, RBI), 5-hydroxytryptamine creatinine sulfate (Wako), (−)-isoprenaline hydrochloride (Sigma), L-nitroarginine methylester hydrochloride (L-NAME) (Sigma), oxytocin (Peptide Institute), prostaglandin $F_{2\alpha}$ (Wako), tetrodotoxin (Wako), verapamil hydrochloride (Wako). 2*a*-[4-(4-phenyl-1,2,3,6-tetrahydropyridyl)butyl]-2*a*,3,4,5-tetrahydro-benzo[*cd*]indol-2(1*H*)-one (DR4004), a selective 5-HT_7 receptor antagonist (Kikuchi et al., 1999), was kindly donated by Meiji Seika (Yokohama, Japan). Drugs except for DR4004, UK14304 and prostaglandin $F_{2\alpha}$ were dissolved in distilled water and added directly to an organ bath. DR4004 and UK14304 were dissolved in dimethylsulfoxide and prostaglandin $F_{2\alpha}$ was dissolved in ethanol, and these solutions were diluted by distilled water or Krebs solution when they were used. The maximum concentrations of dimethylsulfoxide and ethanol in the bath solution were set below 0.2% and 0.1% in the present experiments. Neither 0.2% dimethylsulfoxide nor 0.1% ethanol affected the amplitude or frequency of spontaneous contraction in the uteri of pregnant and nonpregnant pigs.

2.4. Statistical analysis

The results of the experiments are expressed as means ± S.E.M. of at least three preparations obtained from different pigs. Unpaired *t*-test was employed for statistical comparison of the parameters of spontaneous contraction (force, frequency and duration), the inhibition of spontaneous contraction (isoprenaline and 5-hydroxytryptamine) and the force of contractile responses (acetylcholine,

UK14304, oxytocin and prostaglandin $F_{2\alpha}$) between nonpregnant and pregnant uteri. On the other hand, paired *t*-test was used to evaluate effects of L-NAME and DR4004 on the amplitude and frequency of spontaneous contraction in nonpregnant and pregnant porcine uteri. A P value of 0.05 or less was considered statistically significant.

3. Results

3.1. Comparison of spontaneous contractile activities

After equilibration, all LM and CM layers of the uteri from nonpregnant pigs exhibited spontaneous contractile activity. The incubation time required for initiation of spontaneous contraction after suspending in the bath was 13.6 ± 2.4 min (n = 22) for LM and 3.6 ± 0.6 min (n = 23) for CM. The frequency of the spontaneous contraction of the CM (18 ± 0.74/10 min, n = 22) was higher than that of the LM (8.12 ± 0.52/10 min, n = 24), but the duration of one spontaneous contraction in the CM (32 ± 1.7 s, n = 18) was shorter than that in the LM (51 ± 1.9 s, n = 15). In contrast to the frequency and duration, there was no difference between the forces of spontaneous contraction in the LM (2.65 ± 0.21 N/g tissue wet weight, n = 12) and CM (3.22 ± 0.21 N/g tissue wet weight, n = 12) (Fig. 1). Although CM of the uteri from pregnant pigs also showed the spontaneous contractile activities, some uterine LM strips (11 of 23 preparations from 16 pregnant pigs) did not contract spontaneously (see Materials and methods). Of the spontaneously contracting LM and CM from the pregnant uterus, long equilibration time (LM = 34 ± 5.0 min, n = 11; CM = 20.5 ± 3.0 min, n = 13) was required to initiate the spontaneous contraction compared with the nonpregnant porcine uterus. In the CM of the uteri of pregnant pigs, frequency of the spontaneous contraction was significantly lower (5.2 ± 0.48/10 min, n = 16) and the duration considerably longer (97 ± 7.4 s, n = 16) than those in the CM from uteri of nonpregnant pigs (Fig. 1). The force of spontaneous contraction in the uteri of pregnant pigs (3.19 ± 0.39 N/g tissue wet weight, n = 16) was the same as that in the uteri of nonpregnant pigs. Similar with the case of CM layers, in the spontaneously contracting uterine LM of the pregnant pigs, the frequency was significantly lower (4.9 ± 0.8/10 min, n = 12) and the duration was longer (80 ± 8.0 s, n = 12) than those in the LM of nonpregnant pigs. The force of spontaneous contraction increased significantly (4.95 ± 0.31 N/g tissue wet weight, n = 12) in the LM of pregnant pigs (Fig. 1). Tetrodotoxin (1 μM), a neuron blocker, failed to change (n = 3), but verapamil (10 μM) (n = 3) or incubation in the Ca^{2+}-free solution (n = 3) abolished the spontaneous contraction of the LM and CM strips from the uteri of both nonpregnant and pregnant pigs.

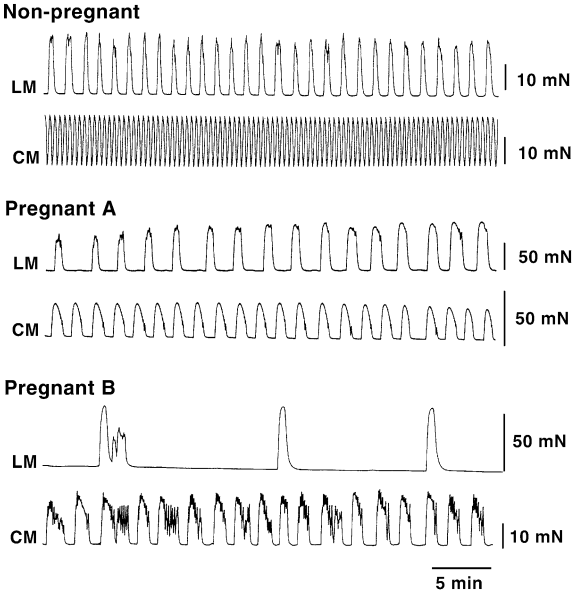

Fig. 1. Typical spontaneous contractions of the longitudinal muscle (LM) and circular muscle (CM) of uteri isolated from a nonpregnant (proestrus) pig and two pregnant pigs (A and B).

Table 1
Effects of pretreatment with L-nitroarginine methylester (L-NAME, 100 μM) and DR4004 (100 nM) on the amplitude and frequency of spontaneous contractions in longitudinal and circular muscles isolated from uteri of nonpregnant and pregnant pigs

	Relative amplitude (%)[a]	Frequency (10 min^{-1})	
	(Control = 100%)	Control	Treatment
L-NAME (100 μM)			
Nonpregnant			
LM	99 ± 1.6 (5)	6.3 ± 0.5 (5)	6.5 ± 0.8 (5)
CM	95 ± 1.6 (5)	19 ± 1.3 (5)	22 ± 2.6 (5)
Pregnant			
LM	110 ± 1.0[b] (7)	3.8 ± 0.8 (7)	4.4 ± 0.7 (7)
CM	113 ± 3.2[b] (11)	2.8 ± 0.3 (11)	3.3 ± 0.4 (11)
DR4004 (100 nM)			
Nonpregnant			
LM	101 ± 1.5 (5)	7.7 ± 0.9 (5)	6.5 ± 0.6 (5)
CM	100 ± 1.8 (5)	19.6 ± 0.7 (5)	19.2 ± 0.9 (5)
Pregnant			
LM	120 ± 3.6[b] (3)	3.3 ± 0.5 (3)	3.1 ± 0.4 (3)
CM	108 ± 3.3[b] (8)	4.6 ± 1.0 (8)	4.6 ± 0.9 (8)

Values are means ± S.E.M. (number of preparations in parentheses).
LM: longitudinal muscle; CM: circular muscle.
[a] Relative amplitude of spontaneous contractions is expressed as a percentage of the control response in the absence of L-NAME or DR4004.
[b] P < 0.05, significantly different from the control values obtained before treatment with L-NAME and DR4004 (paired *t*-test was used for statistical comparison).

The effects of L-NAME and DR4004 on spontaneous contraction were examined to determine whether 5-hydroxytryptamine and/or nitric oxide participate in the change of spontaneous contraction pattern in the pregnant uteri. L-NAME (100 μM for 20 min) did not modify the amplitude or frequency of spontaneous contraction in the uteri of nonpregnant pigs. On the other hand, L-NAME slightly but significantly increased the amplitude of spontaneous

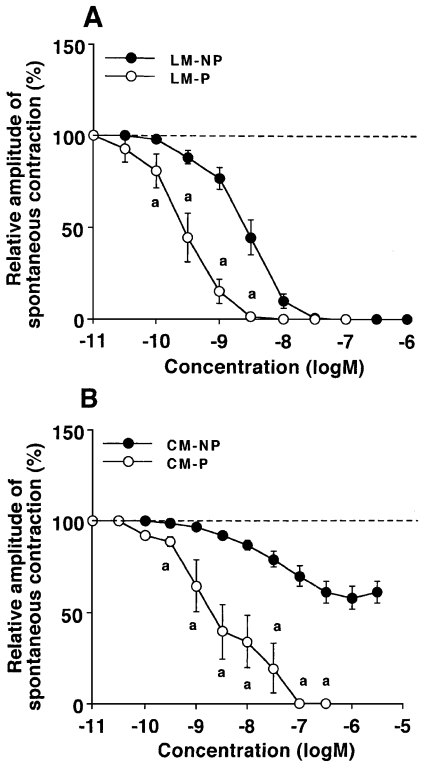

Fig. 2. Pregnancy-associated increase in the inhibitory responses to isoprenaline in porcine myometrial strips. Each symbol shows the concentration–response curves for isoprenaline in the longitudinal (LM, A) and circular muscle (CM, B) strips isolated from uteri of nonpregnant (NP, ●) and pregnant (P, ○) pigs. Ordinate, amplitude of the spontaneous contraction expressed as a percentage of that before treatment with isoprenaline. Abscissa, concentration of isoprenaline ($-\log M$). Points represent the means of four or more preparations with S.E.M. shown by a vertical line. $^{a}P<0.05$, significantly different from the corresponding values for the nonpregnant pigs (unpaired *t*-test was used for statistical comparison).

contraction in the uteri of pregnant pigs (LM = 110 ± 1.0%, $n=7$; CM = 113 ± 3.2%, $n=11$), but its effect on frequency was not marked. DR4004 (100 nM for 20 min) did not affect the amplitude or frequency of spontaneous contractions in the nonpregnant pigs but increased the amplitude of the LM (120 ± 3.6%, $n=3$) and the CM (108 ± 3.3%, $n=8$) in the uteri of pregnant pigs without affecting the frequency (Table 1).

3.2. Inhibition of spontaneous contraction by isoprenaline

Isoprenaline inhibited the spontaneous contraction of both LM and CM layers of the uteri from nonpregnant pigs. $-\text{LogIC}_{50}$ and the maximum inhibition were 8.6 ± 0.1 and 100 ± 0% ($n=9$) in the LM and 6.9 ± 0.17 and 42 ± 6.8% ($n=10$) in the CM, respectively. Isoprenaline also reduced the spontaneous contractile activity in the LM and CM of the pregnant pigs and the $-\log IC_{50}$ was significantly increased in both muscle layers (LM = 9.5 ± 0.11, $n=6$; CM = 8.5 ± 0.26, $n=6$) (Fig. 2). The increase in responsiveness was marked in the CM, and difference between $-\log IC_{50}$ in the LM and that in the CM decreased in the myometrial preparations of pregnant pigs (nonpregnant: 1.7; pregnant: 1.0). In addition, isoprenaline (100–300 nM) abolished the spontaneous contractile activity even in the CM of the pregnant uteri, as it did in the LM layers (Fig. 2).

3.3. Inhibition of spontaneous contraction by 5-hydroxytryptamine

As previously demonstrated (Kitazawa et al., 2000, 2001b), 5-hydroxytryptamine caused a muscle layer-dependent inhibition of the spontaneous contraction in the uteri of nonpregnant pigs. In contrast to the inhibition by isoprenaline, the CM was more sensitive to 5-hydroxytryptamine than was the LM. The $-\log IC_{50}$ value in the LM (6.5 ± 0.12, $n=11$) was significantly lower than that in the CM (7.4 ± 0.1, $n=10$), and the inhibition by 5-hydroxytryptamine was not complete in the LM strips (maximum

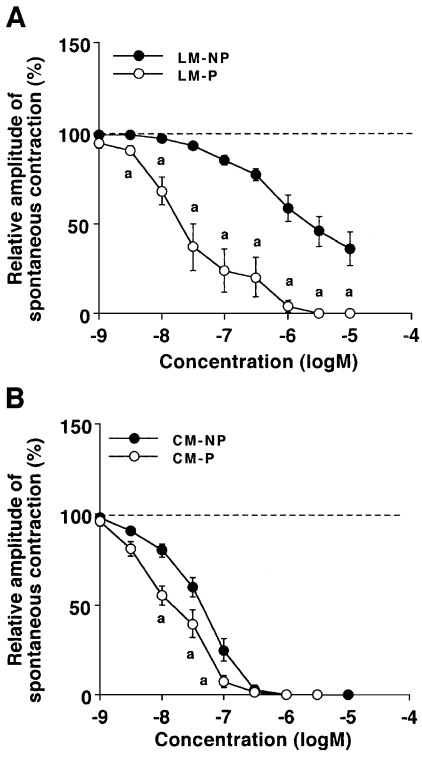

Fig. 3. Comparison of inhibitory responses to 5-hydroxytryptamine in the myometria of nonpregnant and pregnant pigs. Each symbol shows the concentration–response curves for 5-hydroxytryptamine in longitudinal (LM, A) and circular muscle (CM, B) strips of uteri from nonpregnant (NP, ●) and pregnant (P, ○) pigs. Ordinate, amplitude of the spontaneous contraction expressed as a percentage of that before treatment with 5-hydroxytryptamine. Abscissa, concentration of 5-hydroxytryptamine ($-\log M$). Points represent the means of four or more preparations with S.E.M. shown by a vertical line. $^{a}P<0.05$, significantly different from the corresponding values for the nonpregnant pigs (unpaired *t*-test was used for statistical comparison).

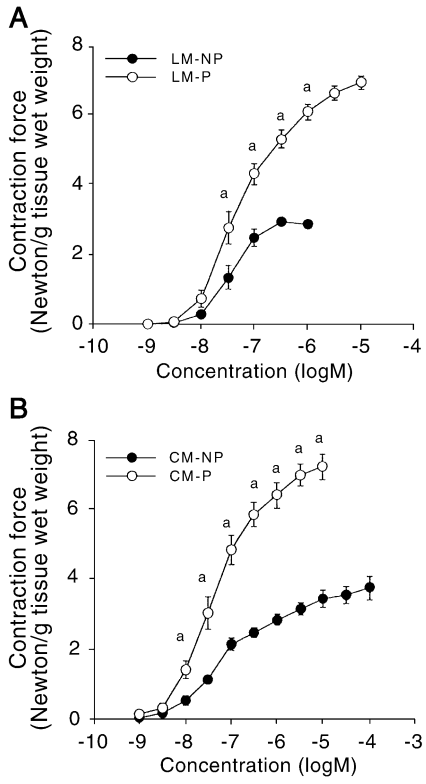

Fig. 4. Comparison of contractile responses to acetylcholine in uteri from nonpregnant and pregnant pigs. Concentration–response curves for acetylcholine in longitudinal (LM, A) and circular muscle (CM, B) strips isolated from uteri of nonpregnant (NP, ●) and pregnant (P, ○) pigs. Ordinate, acetylcholine-induced response is expressed as force of contraction (N/g tissue wet weight) elicited in the muscle preparations. Abscissa, concentration of acetylcholine ($-\log M$). Points represent the means of four or more preparations with S.E.M. shown by a vertical line. [a]$P<0.05$, significantly different from the corresponding values for the nonpregnant pigs (unpaired t-test was used for statistical comparison).

inhibition = 64 ± 9.5%, $n = 11$) (Fig. 3). 5-Hydroxytryptamine inhibited the spontaneous contractions of the LM from uteri of pregnant pigs ($-\log IC_{50} = 7.5 \pm 0.27$, $n = 8$) and eventually abolished the spontaneous contraction in all preparations examined (100 nM–1 μM). The responsiveness to 5-hydroxytryptamine in the CM also increased during pregnancy ($-\log IC_{50} = 7.7 \pm 0.12$, $n = 11$), and the muscle layer-dependent difference in responsiveness to 5-hydroxytryptamine was markedly decreased in the pregnant pigs (difference between $-\log IC_{50}$ in the LM and that in the CM, nonpregnant: 0.9, pregnant: 0.2) (Fig. 3).

3.4. Contractile responses to acetylcholine

Acetylcholine applied to the organ bath caused concentration-dependent contractions in the LM and CM layers from the uteri of nonpregnant pigs. $-\text{LogEC}_{50}$ and maximum force of contractions were 7.4 ± 0.05 and 2.81 ± 0.07 N/g tissue wet weight ($n = 8$) in the LM, and 6.9 ± 0.1 and 3.64 ± 0.33 N/g tissue wet weight ($n = 5$) in the CM. Acetylcholine also contracted both muscle layers from the uteri of pregnant pigs. $-\text{LogEC}_{50}$ and the maximum contraction force were 7.3 ± 0.1 and 6.82 ± 0.2 N/g tissue wet weight ($n = 13$) in the LM, and 7.2 ± 0.13 and 7.06 ± 0.37 N/g tissue wet weight ($n = 11$) in the CM, respectively (Fig. 4). Although the force of maximum contraction by acetylcholine increased in both myometria (increment, LM=2.43, CM=1.94) in pregnant pigs, pregnancy-associated change in $-\log EC_{50}$ values was not so marked (slight increase in the CM) (Fig. 4). The high-K^+-induced contraction was also compared between nonpregnant and pregnant uteri to clarify whether increase in the contractile force was specific to acetylcholine or not. The forces of the 50 mM high-K^+-induced contraction in the LM strips were 2.68±0.21 N/g tissue wet weight (n=9) for the nonpregnant pigs and 5.67±0.5 N/g tissue wet weight (n=11) for the pregnant pigs. In the CM strips, the forces of the high-K^+-induced contraction were also increased (nonpregnant: 2.81±0.29 N/g tissue wet weight, n=8; pregnant: 4.98±0.63 N/g tissue wet weight, n=8). The increments (pregnant/nonpregnant) of the responses to high-K^+ (LM=2.12, CM=1.77) were comparable to those of acetylcholine.

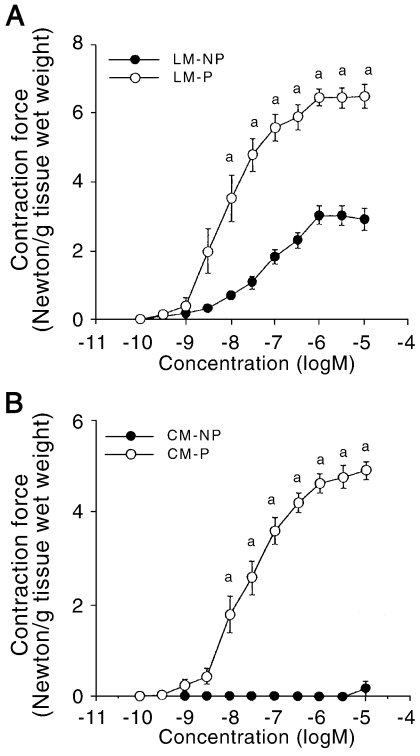

Fig. 5. Increase in the responsiveness to UK14304 in uteri from pregnant pigs. Effects of UK14304 on longitudinal (LM, A) and circular muscles (CM, B) of uteri from nonpregnant (NP, ●) and pregnant (P, ○) pigs were examined, and the concentration–response curves were shown. Ordinate, UK14304-induced response is expressed as force of contraction (N/g tissue wet weight) elicited in the muscle preparations. Abscissa, concentration of UK14304 ($-\log M$). Points represent the means of four or more preparations with S.E.M. shown by a vertical line. [a]$P<0.05$, significantly different from the corresponding values for the nonpregnant pigs (unpaired t-test was used for statistical comparison).

Table 2
Comparison of the contractile responses to oxytocin and prostaglandin $F_{2\alpha}$ in the longitudinal and circular muscles isolated from uteri of the nonpregnant and pregnant pigs

	Contractile force (N/g tissue wet weight)					
	0.1 nM	1 nM	10 nM	100 nM	1 μM	10 μM
Oxytocin						
Longitudinal muscle						
Nonpregnant	0.15 ± 0.15 (5)	2.49 ± 0.15 (5)	2.73 ± 0.06 (5)	2.81 ± 0.09 (5)		
Pregnant	2.25 ± 0.61[a] (4)	4.97 ± 0.37[a] (4)	6.57 ± 0.15[a] (4)	7.62 ± 0.69[a] (3)		
Circular muscle						
Nonpregnant	0 ± 0 (5)	0.08 ± 0.05 (5)	0.56 ± 0.15 (5)	1.0 ± 0.31 (5)		
Pregnant	1.53 ± 0.76[a] (4)	2.99 ± 0.56[a] (5)	4.52 ± 0.37[a] (5)	5.27 ± 0.23[a] (3)		
Prostaglandin $F_{2\alpha}$						
Longitudinal muscle						
Nonpregnant				2.78 ± 0.09 (5)	2.85 ± 0.11 (5)	
Pregnant				5.32 ± 0.61[a] (4)	6.54 ± 0.44[a] (4)	
Circular muscle						
Nonpregnant				0.01 ± 0.01 (7)	0.04 ± 0.04 (7)	− 0.15 ± 0.06 (7)
Pregnant				3.47 ± 0.44[a] (6)	5.2 ± 0.67[a] (5)	6.33 ± 0.47[a] (4)

Values are means ± S.E.M. (number of preparations in parentheses).
[a] $P < 0.05$, significantly different from the corresponding values for the nonpregnant pigs (unpaired *t*-test was used for statistical comparison).

3.5. Contractile response to UK14304

UK14304 (1 nM–10 μM), an α_2-adrenoceptor agonist, caused contraction of the LM strips ($-\log EC_{50} = 7.2 \pm 0.1$, maximum contraction = 3.00 ± 0.26 N/g tissue wet weight, $n = 5$) but did not change the amplitude of spontaneous contraction and tonus of the CM strips in uteri of nonpregnant pigs (Fig. 5) as was previously reported (Taneike et al., 1994). The effects of UK14304 in LM and CM strips isolated from uteri of pregnant pigs were also investigated. In the uterine LM of pregnant pigs, both $-\log EC_{50}$ and the force of the maximum contraction by UK14304 were significantly increased (8.1 ± 0.22 and 6.41 ± 0.34 N/g tissue wet weight, $n = 9$). Pregnancy-associated change in contractile force of the LM (increment = 2.14) was comparable to acetylcholine and high-K^+. UK14304 caused a concentration-dependent contraction of the CM of pregnant pigs quite different from the CM of nonpregnant pigs. $-\text{LogEC}_{50}$ and maximum contraction force were 7.5 ± 0.12 and 4.8 ± 0.21 N/g tissue wet weight ($n = 10$) (Fig. 5).

3.6. Contractile responses to oxytocin and prostaglandin $F_{2\alpha}$

Oxytocin (0.1–100 nM) caused a contraction of myometria from both pregnant and nonpregnant pigs. Table 2 shows the pregnancy-related changes in oxytocin-induced contraction of the LM and CM layers. In the nonpregnant pigs, the response to oxytocin was muscle layer-dependent, and the LM was more sensitive than was the CM. Pregnancy significantly increased the contractile responses to oxytocin in both muscle layers, and the degree of pregnancy-associated increase in the contractile responses was marked in the CM.

Prostaglandin $F_{2\alpha}$ contracted the LM layers from uteri of nonpregnant pigs, but the CM layers were insensitive to prostaglandin $F_{2\alpha}$, though slight relaxation was caused at 10 μM. In the uteri of pregnant pigs, prostaglandin $F_{2\alpha}$ contracted the CM layers, and the contractile force at 10 μM prostaglandin $F_{2\alpha}$ was 6.33 ± 0.47 N/g tissue weight ($n = 4$). Of the responses to prostaglandin $F_{2\alpha}$ in the LM layers, contractile force also increased significantly in the uteri of pregnant pigs (Table 2).

4. Discussion

This is a first in vitro functional study in which the spontaneous contractility and drug responsiveness of the myometrium in nonpregnant pigs was compared with those of myometrium from pregnant pigs. Slow development of myometrial contractility, and less frequent but large force and long duration of spontaneous contraction were characteristics of the uteri of pregnant pigs. On the responses to bioactive substances, increase in responsiveness to both inhibitory (isoprenaline and 5-hydroxytryptamine) and contractile agents (acetylcholine, UK14304, oxytocin and prostaglandin $F_{2\alpha}$) were observed. Increment was conspicuous in the myometrial layer that was less sensitive to each agonist in the nonpregnant pigs. Therefore, the myometrial layer-dependent difference in responsiveness to bioactive substances in the pregnant pigs was less marked than that observed in the nonpregnant pigs.

The LM and CM strips from the uteri of nonpregnant pigs exhibited spontaneous contractile activity in a muscle layer-dependent manner (CM>LM) as was previously reported (Kitazawa et al., 1998, 2000). Although all of the CM preparations isolated from pregnant pigs contracted spontaneously, some LM preparations (11 of 23 preparations from 16 pregnant pigs) did not show spontaneous contractions. Comparing the spontaneous contraction pro-

files, decrease in the frequency, increase in force and slow contraction (increase in duration of one spontaneous contraction) were observed in the uteri of pregnant pigs. These low frequency, and slow and large contraction kinetics of the uterus in pregnant pigs might be more effective in initiating labor. Pregnancy-related decrease in frequency of the spontaneous contraction was inconsistent with that demonstrated in the rat (Ohashi et al., 1996), suggesting that there is a species-dependent pattern in the change of spontaneous activity by the pregnancy. However, slow kinetics of myometrial contraction during pregnancy has already been reported in humans (Tchirikov et al., 2000). The fact that verapamil and Ca^{2+}-free solution abolished the spontaneous contractility in the uteri from the nonpregnant and pregnant pigs suggests that extracellular Ca^{2+} and voltage-dependent Ca^{2+} channels were important for initiation of spontaneous contraction without regarding nonpregnant or pregnant pigs. Gestational stage-dependent inhibitory action of L-arginine and pregnancy-dependent upregulation of nitric oxide synthase expression in the uterus (Yallampalli et al., 1994; Buhimschi et al., 1995; Norman et al., 1999) indicate that the nitric oxide–guanylate cyclase–cGMP system plays an important role in the pregnancy-related changes of uterine motility. In the present study, L-NAME did not modify the spontaneous contraction in the nonpregnant uterus as was previously reported (Kitazawa et al., 1998), but the amplitude of spontaneous contraction in the LM and CM from uteri of pregnant pigs was increased by L-NAME, suggesting that nitric oxide contributes to the pregnancy-related change of uterine motility. The 5-HT_7 receptor has been reported to be present in the porcine uterus and to mediate the 5-hydroxytryptamine-induced inhibition of uterine contractility (Kitazawa et al., 1998, 2000). DR4004, a potent and selective 5-HT_7 receptor antagonist (Kikuchi et al., 1999), had no effect in the uteri of nonpregnant pigs but increased the amplitude of spontaneous contraction in the pregnant pigs. These results suggest the involvement of 5-HT_7 receptor in changes of spontaneous contractility in the pregnant pigs. The fact that sensitivity of 5-hydroxytryptamine increased in both myometria of pregnant pigs indicates that 5-hydroxytryptamine and 5-HT_7 receptor-mediated inhibition becomes more functional during pregnancy. Histochemical and biochemical studies have demonstrated that mast cells (one source of 5-hydroxytryptamine) in the uteri of mice and humans increase in number during pregnancy (Padilla et al., 1990; Mori et al., 1997). The increase in the source of 5-hydroxytryptamine and the responsiveness to 5-hydroxytryptamine (present study) support the assumption that 5-hydroxytryptamine is involved in regulation of uterine motility in pregnant pigs. In the uteri of the pregnant pigs, significant long equilibration time was needed to initiate the spontaneous contraction and some LM strips did not show spontaneous contraction. The mechanisms of these phenomena were not clear at present, but an activation of endogenous inhibitory pathways and slow contraction kinetics of uterine smooth muscle itself suggested in the present study might explain the phenomena.

Isoprenaline also inhibited the spontaneous contraction of the myometria of nonpregnant and pregnant pigs. As was previously reported, LM was more sensitive than was CM in the nonpregnant pigs, because of the heterogeneous distribution of β_2-adrenoceptors (receptor density, LM:CM=3.3:1) (Kitazawa et al., 2001b). In the present experiment, the isoprenaline-induced inhibition was increased in both myometria of pregnant pigs. Pregnancy-related increase in density of β-adrenoceptors and pregnancy-related increase in adenylate cyclase activity have already been demonstrated in the rat and are considered to be important for facilitation of uterine quiescence during pregnancy (Cohen-Tannoudji et al., 1991; Engstrom et al., 1997; Lindeman et al., 2000). Similar upregulation in β_2-adrenoceptors in the porcine uterus could explain the high responsiveness to isoprenaline. Different from change in the responses to 5-hydroxytryptamine, the pregnancy-dependent increase in isoprenaline-induced inhibition was marked in the CM, which was less sensitive in the nonpregnant pigs. As a consequence, myometrial layer-dependent increase in responsiveness to 5-hydroxytryptamine (LM>CM) and isoprenaline (CM>LM) decreases the difference in the responsiveness of inhibitory agents between LM and CM.

Kim et al. (1998) investigated the force-generating capacities of myometria isolated from nonpregnant and pregnant rats, and found that the forces of contraction by high-K^+ and several receptor agonists (oxytocin, carbachol, prostaglandin E_2 and prostaglandin $F_{2\alpha}$) were two or three times greater in the pregnant rat uterus than those in the nonpregnant rat uterus. Degree of Ca^{2+}-dependent myosin light chain phosphorylation and an increase in actin contents in the myometria isolated from the pregnant rats have been reported as the difference between nonpregnant and pregnant rat uteri. Similar increases in the contractile force of high-K^+, acetylcholine, UK14304, oxytocin and prostaglandin $F_{2\alpha}$ were observed in the uteri of pregnant pigs. The mechanisms underlying the pregnancy-related increase in the present study were not clear. However, because increment of acetylcholine- and UK14304-induced contractions was almost consistent with that of high-K^+, upregulation of voltage-dependent Ca^{2+} channels, increase in efficiency of channel opening and supersensitivity of contractile apparatus to Ca^{2+} were speculated as possible mechanisms for pregnancy-related nonselective increase of contractile responses. It is accepted that pregnancy changes the expression of receptors, such as oxytocin receptor (Fuchs et al., 1982, 1984), prostanoids FP receptor (Dong and Yallampalli, 2000) and α_2-adrenoceptor (Arkinstall and Jones, 1988). Therefore, increase in expression of contractile receptors in the uteri of pregnant pigs was suggested to be another mechanism for the pregnancy-associated increase in the contractile responses.

We have already reported muscle layer-dependent differences (LM>CM) in the responsiveness to uterotonic agents (acetylcholine, UK14304, histamine, oxytocin, endothelin

and prostaglandin $F_{2\alpha}$) and suggested that muscle layer-dependent difference of uterotonic agents is a character in the uteri of nonpregnant pigs (Taneike et al., 1994, 1995; Kitazawa et al., 1997, 2001a; Isaka et al., 2000; Cao et al., 2002). In the present study, an increase in responsiveness to all uterotonic agents was marked in the CM layers and the muscle layer-dependent difference observed in the uteri from nonpregnant pigs became less remarkable in the uteri of the pregnant pigs. Although sex steroid hormones (progesterone and estrogen) have been demonstrated to modulate the expression of FP prostanoid (Dong and Yallampalli, 2000) or oxytocin receptors (Fuchs et al., 1984), clarification of the hormone mechanisms underlying the pregnancy-associated changes in the pig uterus is beyond the scope of the present experiments. However, in the first half stage of pregnancy in pigs (0–60 days of gestation), it was reported that plasma progesterone was maintained at a high level but that the estradiol 17-β level was undetectable (Anderson, 1987; Robertson and King, 1974). Therefore, progesterone might regulate the expression of the respective receptors in the pig and affect the responsiveness of the myometrium to bioactive substances.

In conclusion, slow development of mechanical activity, low frequency but large and slow kinetics of spontaneous contractions are characteristics of the pregnant pig uteri, and nitric oxide- and 5-hydroxytryptamine-mediated inhibition is supposed to be partially involved in the pregnancy-related change in spontaneous contractions. The inhibition of spontaneous contraction by isoprenaline and 5-hydroxytryptamine increased in the pregnant pigs. Of the responses to uterotonic agents, both contractile force and responsiveness to acetylcholine, UK14304, oxytocin and prostaglandin $F_{2\alpha}$ were also increased in the uteri of pregnant pigs. Increment of the responses to both inhibitory and contractile substances was marked in the muscle layer that was less sensitive to each substance in the uteri of nonpregnant pigs. Due to the pregnancy-associated changes, muscle layer-related different responsiveness observed in the nonpregnant pigs decreased in the pregnant pigs.

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