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Cigarette smoke extract enhances oxytocin-induced rhythmic contractions of rat and human preterm myometrium

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Abstract

Although smoking during pregnancy is a major risk factor for preterm delivery, the underlying mechanism by which smoking stimulates uterine contractions is still poorly understood. In the present study, we tried to clarify the effects of smoking on myometrial contractility induced by oxytocin (OT) using cigarette smoke extract (CSE). Myometrial strips, which were taken from the rat on day 16 of pregnancy, and from human preterm and term delivery groups, were incubated overnight with several doses of CSE at 37 °C under non-hormonal conditions. The uterine contractile sensitivity and activity (force and frequency) upon exposure to OT were investigated. Furthermore, the expression levels of oxytocin receptor (OTR) mRNA in the myometrial strips were investigated by real-time PCR. Contractile sensitivity to OT in the rat CSE (10^{-7} pieces/ml) group was found to be significantly higher than in the control group ($P<0.05$). Contractile activity did not differ between the CSE and control groups. The expression levels of rat OTR mRNA in the CSE (10^{-7} pieces/ml) group were significantly higher than in the control group ($P<0.01$). Similarly, in preterm myometrial strips, the expression levels of human OTR mRNA in the CSE (10^{-7} pieces/ml) group were significantly higher than in the control group ($P<0.05$). These findings suggest that CSE directly increases the contractile sensitivity of preterm myometrium in response to OT by upregulating the expression of OTR mRNA and thereby increases the risk of preterm delivery in women, who smoke during pregnancy.

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Introduction

Smoking during pregnancy is known worldwide to be one of the most important causes of fetal death and perinatal mortality and morbidity. The incidences of spontaneous abortion, preterm delivery, and low birth weight infants among mothers, who smoke during pregnancy, are significantly higher than among non-smokers (Andres & Day 2000). In 1957, Simpson (1957) first suggested an association between smoking and preterm delivery and identified a dose–response relationship between cigarette smoking and preterm delivery; he also reported that the incidence of low birth weight infants among smokers was nearly twice than among non-smokers. Other investigators have also reported similar conclusions (Meyer & Tonascia 1977, McDonald *et al.* 1992, Meis *et al.* 1995).

Clinical and experimental evidences have indicated that the primary pathogenic mechanisms involved in preterm labor are (1) activation of the hypothalamus–pituitary–adrenal axes of the mother and fetus; (2) inflammation of the amnion, chorion, and deciduas; (3) decidual hemorrhage; and (4) pathologic distension of the uterine myometrium. They share a common final pathway resulting in myometrial contraction. It is well known that both oxytocin (OT) and prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$) induce pregnant uterine contractions. These agents have been used clinically for the induction of delivery or termination of pregnancy. OT and PG $F_{2\alpha}$ induce myometrial contractions via the oxytocin receptor (OTR) and PG $F_{2\alpha}$ receptor (FP) respectively, and pathways activated by the binding of OT to the OTR and PG $F_{2\alpha}$ to the FP are involved in preterm labor and delivery (Bernal *et al.* 1993). Prostaglandin synthesis inhibitors (e.g., indomethacin) inhibit spontaneous uterine contractions and can be used for the treatment of preterm labor and delivery (Keirse 1992). Additionally, the OT antagonist, atosiban, reduces uterine contractility in women with threatened preterm delivery (Akerlund *et al.* 1987, Moutquin *et al.* 2000). FP gene expression has been detected in both pregnant and non-pregnant human myometria, although the

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level of expression in the pregnant myometrium is less than that in the non-pregnant myometrium (Matsumoto *et al.* 1997). On the other hand, the expression of human OTR mRNA in the myometrium is increased during pregnancy, being more than 300-fold higher at parturition than in non-pregnant myometrium (Kimura *et al.* 1996). Additionally, it has been reported that contractile sensitivity to OT is significantly increased in a preterm delivery group (<37 weeks), but reduced in a postterm delivery group in comparison with the term delivery group (Takahashi *et al.* 1980). These reports suggest that the increased risk of preterm labor and delivery in smokers is related to increased contractile sensitivity and activity of the uterine myometrium upon exposure to OT and/or PG $F_{2\alpha}$ through the respective receptors.

We have recently reported that 3 days of inhalation of cigarette smoke by pregnant rats (days 14–16) increases contractile sensitivity and activity of the myometrium upon exposure to OT, but not to PG $F_{2\alpha}$ (Egawa *et al.* 2003). Additionally, we have indicated that the expression of OTR mRNA, but not FP mRNA, in rat myometrium is increased by the inhalation of cigarette smoke (Egawa *et al.* 2003). Therefore, the increased contractile sensitivity and activity of pregnant myometrium in response to OT may result from the increased expression of OTR mRNA caused by smoking and increase the risk of preterm delivery in smokers. However, it remains unclear whether the effects of inhalation of cigarette smoke on the expression of OTR mRNA are a direct action on the myometrium or an indirect action via feto–maternal hormonal conditions influenced by cigarette smoke. This uncertainty is based upon the findings that progesterone decreases the levels of uterine OTR mRNA and protein in pregnant rats (Soloff *et al.* 1983), and estrogen increases the levels of OTR in the rat uterus and in human-cultured myometrial cells (Soloff 1975, Adachi & Oku 1995). Additionally, it has been reported that OTR levels are upregulated through progesterone regression, which occurs during luteolysis in the ewe (Hixon & Flint 1987, Zhang *et al.* 1992). Therefore, the increased expression of OTR mRNA in the pregnant rat myometrium in the smoking group may be caused indirectly through progesterone regression via luteolysis. Thus, inhalation of cigarette smoke may damage the function of corpus luteum in the pregnant rat.

In the present study, we treated rat myometrial strips with cigarette smoke extract (CSE) under non-hormonal conditions to evaluate whether the effects of inhaled cigarette smoke are due to direct action on the pregnant rat myometrium, and investigated the contractile response to OT and the expression of OTR mRNA in the myometrium. Further, we evaluated the effect of CSE on myometrial contraction in pregnant human myometrium using the same non-hormonal system.

Materials and Methods

Chemicals

Cigarettes of a domestic brand (Mild Seven) with filters were purchased from Japan Tobacco (Tokyo, Japan). Atonin-O (OT) was obtained from Teizo (Tokyo, Japan). PG $F_{2\alpha}$ was obtained from Ono Pharmaceutical Co. Ltd (Osaka, Japan). Atosiban, a specific OT antagonist, was donated by Ferring AB (Limhamn, Sweden). TRIzol reagent, phenol red-free Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, random primers, DNase I amplification grade and 10× DNase I buffer were purchased from Invitrogen. Recombinant Taq DNA polymerase gene Taq, 10× Gene Taq universal buffer, and RNase inhibitor were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan). ReverTra Ace-α-kit (ReverTra Ace reverse transcriptase, dNTP mixture, 5× RT buffer) and anti-Taq high were purchased from Toyobo Co. Ltd (Tokyo, Japan). SYBR green I nucleic acid gel stain was purchased from Roche.

Animals

Pregnant Wistar rats, obtained from Shimizu Laboratory Supplies (Kyoto, Japan), housed under controlled conditions (12 h light:12 h darkness photoperiod) were provided with water and rat chow *ad libitum*. They were euthanized under ether anesthesia on day 16 of pregnancy, after which the uterus was removed and used for the experiments. This study was approved by the Animal Committee of Kansai Medical University and conducted in accordance with the Guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching published by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

Human subjects

This study was performed using a protocol for the protection of human subjects approved by the ethical committee of Kansai Medical University in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. Human myometrial samples for the experiments were obtained from six patients, who had undergone routine abdominal cesarean section from a low-segment transverse incision under epidural anesthesia before the onset of labor pains. Myometrial samples were obtained at 29, 34, and 35 weeks of gestation (preterm group) and at 37, and 38 weeks of gestation (term group). After delivery of the infant and placenta, a sample of myometrium was taken from the upper margin of the lower uterine segment incision using tissue forceps and scissors. The obtained tissue samples were immediately submerged in ice-cold ViaSpan (Belzer UW, Bristol-Myers Squibb Company,

Princeton, NJ, USA), and transported to the laboratory to be used within 24 h of dissection.

Preparation of CSE

CSE was prepared by a previously published method, with modifications (Laurent *et al.* 1983, Yasuda *et al.* 1995). Briefly, the smoke of ten filtered cigarettes, each containing 0.8 mg of nicotine and 10 mg of tar according to the manufacturer's report, were aspirated consecutively through an experimental apparatus with a constant airflow (0.3 l/min) driven by an air compressor. The smoke was bubbled through 10 ml of phenol red-free DMEM/F-12 medium. The obtained CSE was then filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA) to remove bacteria and large particles. The CSE was prepared and diluted to the experimental dose with phenol red-free DMEM/F-12 medium immediately before each experiment, unless when otherwise indicated. The pH of the CSE was between 7.4 and 7.5 after dilution for each experiment.

Preparation of strips

After the myometrium was obtained, the myometrial strips were prepared (width, 2–3 mm; length, 10–15 mm). Overnight incubation was performed with several doses of CSE (final concentration, 10^{-1}–10^{-8} pieces/ml) with phenol red-free DMEM/F-12 medium or with phenol red-free DMEM/F-12 medium alone as a control at 37 °C in humidified atmosphere of 5% CO^2 in air. After the overnight incubation, some samples from these strips were immediately used for the assessment of contractile sensitivity and activity, and others were immediately frozen in liquid nitrogen, and stored at −80 °C until the RNA was extracted.

Contractile sensitivity and activity

The contractile sensitivity and activity of myometrium were evaluated by a previously described method (Egawa *et al.* 2003). Briefly, each strip after the overnight incubation under one of the several conditions was attached to a holder under a 1 g resting tension. After equilibration for 60 min in a physiological saline solution, each strip was repeatedly exposed to 72.7 mM KCl (high K^+) solution until the response 'became stable' was obtained. In the present study, myometrial strips with spontaneous contraction after preloading of the high K^+ solution were excluded, because we could not then confirm whether contractions were caused by the agonist. After preloading of the high K^+ solution, appropriate concentrations of OT or PG $F_{2\alpha}$ were added to evaluate uterine sensitivity and activity. The physiological saline solution contained the following: NaCl (136.9 mM), KCl (5.4 mM), $CaCl_2$ (1.5 mM), $MgCl_2$ (1.0 mM), $NaHCO_3$ (23.8 mM), glucose (5.5 mM), and EDTA (0.01 mM). The high K^+ solution was prepared by replacing NaCl with an equimolar amount of KCl. These solutions were saturated with a 95% O_2/5% CO_2 (v/v) mixture at 37 °C and pH 7.4. Muscle contraction was recorded isometrically with a force–displacement transducer (Model TB611T; Nihon Kohden, Tokyo, Japan) that was connected to a model 3134 strain amplifier and a model 3056 ink-writing recorder (Yokogawa, Tokyo, Japan). The data was simultaneously input into a personal computer (Microsoft Windows 2000 professional). The input data were analyzed with analysis software, Unique Acquisition (Unique Medical Co. Ltd, Tokyo, Japan). We considered the contraction induced by 72.7 mM K^+ solution as the reference response. The amplitude of the high K^+-induced muscle contraction was set at 100%, and the amplitude of the OT-induced contraction was calculated in reference to the amplitude of the high K^+-induced contraction. The OT-induced contractions were counted over a period of 30 min, and their frequency was expressed as the mean value per 10 min. Additionally, atosiban, a specific OT antagonist, was added to the rhythmically contracted myometrial strips.

RNA isolation and DNase I treatment

Total RNA was prepared from each strip by the acid guanidinium phenol–chloroform method using TRIzol reagent and dissolved in the appropriate amount of 0.01% diethyl pyrocarbonate water. The quality of the RNA was measured spectrophotometrically at an absorbance ratio of optical density (OD) 260:OD 280 (absorbance ratio > 1.8), while the quantity was assessed at OD 260. Two micrograms of each RNA sample were incubated for 15 min at room temperature in a volume of 20 μl, containing 2 μl 10× DNase I buffer (200 mM Tris–HCl (pH 8.4), 20 mM $MgCl_2$, 500 mM KCl), 2 μl DNase I amplification grade and 1 μl RNase inhibitor. Following the incubation, the reaction was terminated by the addition of 2 μl 25 mM EDTA solution, and was heated for 10 min at 65 °C to inactivate the enzyme.

RT

RT was performed using the ReverTra Ace-α-Kit, according to the manufacturer's instructions, as follows: 1 μg total RNA treated with DNase I in 20 μl reaction mixture (final concentrations: 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 μM random primer, 1 mM TTP, 0.3 μM (methyl-3H) dTTP, 1 mM dNTP) containing 100 U ReverTra Ace reverse transcriptase and 5× RT buffer at 30 °C for 10 min, at 42 °C for 20 min, followed by inactivation of the enzyme at 99 °C for 5 min with a TaKaRa PCR Thermal Cycler MP (TaKaRa Shuzo, Kyoto, Japan). Finally, the first-strand cDNA was dissolved in 80 μl distilled water and stored at −20 °C. The control

reaction was performed simultaneously under identical conditions, but without reverse transcriptase.

Quantitative real-time PCR analysis with SYBR green I

For quantitation of OTR mRNA and FP mRNA in rat myometrium and human myometrium, real-time quantitative PCR using the SYBR green I nucleic acid gel stain was performed in triplicate on 96-well optical reaction plates (Ina. Optika Co. Ltd, Osaka, Japan) using an iCycler iQ (Bio-Rad). The PCR was performed in a total volume of 25 μl containing 2 μl of the above described solution of cDNA, 1 μl each of the 3′ and 5′ primers (3.75 pmol each), 1 μl $MgCl_2$ (25 mM), 2 μl dNTP (2.5 mM), 2.5 μl 10× Gene Taq universal buffer, 0.375 U recombinant Taq DNA polymerase gene Taq, 0.075 μl MAB for hot start PCR anti-Taq high, and 1/75 000 SYBR green I nucleic acid gel stain. PCR amplification of each sample was performed on the same reaction plate using both the OTR and FP primer pairs together with primers for the elongation factor (EF)-1α gene, which served as an internal control for both rat and human samples. EF-1α is valid as a reference 'housekeeping' gene for transcription profiling and is used for real-time PCR experiments (Frost & Nilsen 2003). The oligonucleotide primers were synthesized by Proligo Japan (Kyoto, Japan). The rat OTR oligonucleotide primer sequences were 5′-AATCCGCACGGTGAAGATGACC-3′ (forward primer) and 5′-ACGAGCAGAGCAGCAGAA-GAAGC-3′ (reverse primer), and these sequences amplified a 241-bp (807–1047) fragment of rat OTR cDNA (Rozen *et al.* 1995). The rat FP oligonucleotide primer sequences were 5′-GAAGTTTAGAAGTCAG-CAGC-3′ (forward primer) and 5′-ACTCAGAGATAG-CAGCAACC-3′ (reverse primer). These sequences amplified a 359-bp (690–1048) fragment of rat FP cDNA (Lake *et al.* 1994). The human OTR oligonucleotide primer sequences were 5′-atgtggagcgtctgggatgc-3′ (forward primer) and 5′-gctcaggacaaaggaggacg-3′ (reverse primer), and these sequences amplified a 237-bp (1508–1744) fragment of human OTR cDNA (Kimura *et al.* 1992). The human FP oligonucleotide primer sequences were 5′-tagtgtctcctgcagctgcg-3′ (forward primer) and 5′- tcaatggccatcacactgcc-3′ (reverse primer), which amplified a 370-bp (177–546) fragment of human FP cDNA (Lake *et al.* 1994). The EF-1α oligonucleotide primer sequences were 5′-TCTGGTTGGAATGGTGA-CAACATGC-3′ (forward) and 5′-AGAGCTTCACT-CAAAGCTTCATGG-3′ (reverse) (Strausberg *et al.* 2002, Nakamoto *et al.* 2005). These sequences amplified a 329-bp fragment of EF-1α cDNA (bases; 595–923). The PCR comprised 50 cycles: 94 °C for 30 s, 72 °C for 60 s (set-point temperature was decreased every two cycles by 0.3 °C), and 72 °C for 30 s. After PCR, a melting curve was constructed by increasing the temperature from 65 to 95 °C with a temperature transition rate of 0.5 °C/30 s. After analysis of the melting curve, the concentration of each sample was calculated from the threshold cycle (Ct). To facilitate a comparison of mRNA expression, the Ct values of OTR or FP from each sample were normalized by EF-1α Ct values obtained from the same sample. The Ct values were averaged from triplicate values. The differences between the mean Ct values of OTR or FP and those of EF-1α were calculated as follows: ΔCt (sample) = Ct (sample) − Ct (EF-1α). The final result was expressed as $2^{-\Delta Ct\ (sample)}$. In the present study, three series of real-time PCR for OTR and PG $F_{2\alpha}$ were performed with myometrial samples taken from individual pregnant rats and pregnant women respectively. One series in the rat included three groups: two CSE groups treated with 10^{-6} and 10^{-7} pieces/ml CSE and a control group. One series in humans included two groups: one CSE group treated with 10^{-7} pieces/ml CSE and a control group. Each group consisted of eight myometrial strips obtained from the same rat or human. To eliminate the possibility of contamination with genomic DNA during extraction of total RNA, a control reaction with each primer pair was performed at the same time under identical conditions without RT. No amplification was detected.

Statistical analysis

Data are expressed as the mean ± S.E.M. The results were analyzed with a statistical software package, StatView version 5.0 (SAS Institute, Inc., Cary, NC, USA). For the data regarding uterine contractile sensitivity and activity and the expression levels of OTR mRNA and FP mRNA, the differences in the measured parameters across the different groups were statistically assessed using one-way ANOVA with repeated measurements, followed by Fisher's protected least significant difference, multiple range test. A level of $P < 0.05$ was considered statistically significant.

Results

Contractile sensitivity to OT of rat myometrial strips treated with CSE

In rat samples treated with low-dose CSE (final concentrations, 10^{-6} and 10^{-7} pieces/ml), spontaneous and rhythmic contractions of the myometrial strips occasionally occurred before stimulation by OT. However, only a few spontaneous contractions occurred in samples from the control and high-dose CSE groups (final concentrations, 10^{-2} and 10^{-3} pieces/ml). Strips having spontaneous contractions were excluded from the analysis. However, our findings indicate that irritability was much greater in the low-dose CSE group than in either the control or high-dose CSE groups. As shown in Fig. 1, contractile sensitivity to OT in the rat myometrial strip treated with low-dose CSE

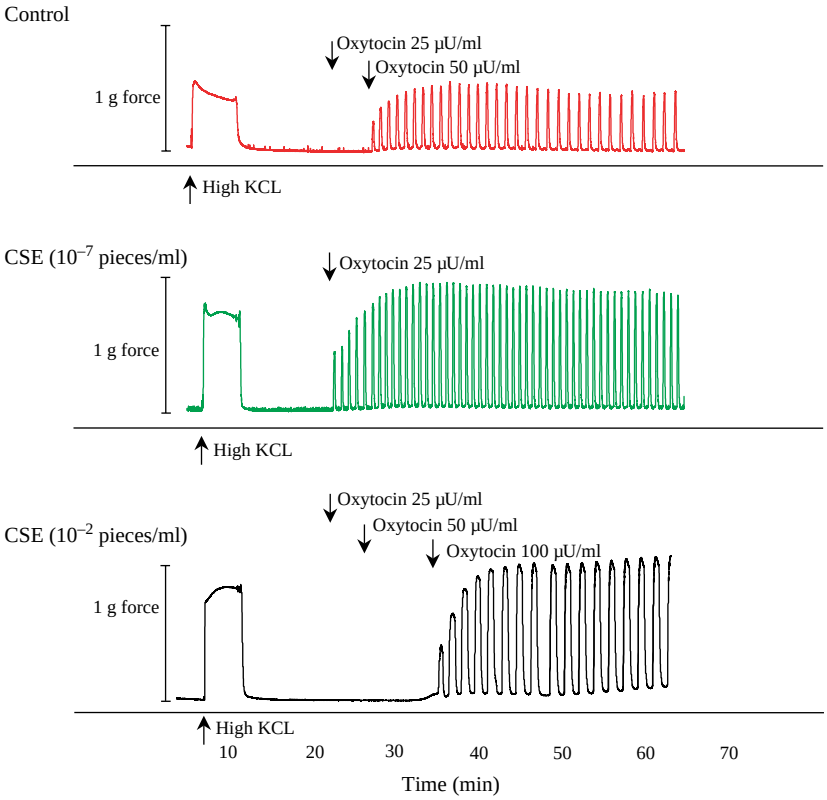

Figure 1 Oxytosin (OT)-induced contractions of rat myometrial strips treated with the vehicle, CSE 10^{-7} pieces/ml and CSE 10^{-2} pieces/ml. Preparation and treatment of myometrial strips are as described in 'Materials and Methods'. Uterine sensitivity was evaluated according to the dose of OT when the first rhythmic myometrial contraction occurred. The control, CSE 10^{-7} pieces/ml, and CSE 10^{-2} pieces/ml strips presented here showed sensitivity to 50, 25, and 100 μU/ml OT respectively.

(10^{-7} pieces/ml) was 25 μU/ml and was higher than in the control strip (50 μU/ml), but the sensitivity (100 μU/ml) in the myometrial strip treated with high-dose CSE (10^{-2} pieces/ml) was lower than in the control strip. Myometrial strips treated with CSE (10^{-1} pieces/ml) were all inactive, and almost half the samples incubated with CSE (10^{-2} pieces/ml) were inactive. In another myometrial strip, the rhythmic contractions that were found in the control and CSE-treated strips after the administration of OT were completely inhibited by the administration of atosiban (final concentration, 10^{-7} M), a specific OT antagonist (Fig. 2).

In the present study, we could not evaluate more than four myometrial strips simultaneously by using our instruments. We usually evaluated the contractile response of 8–12 myometrial strips (four strips×2–3 cycle) in one experiment. Therefore, we separated the experiments into two series: high-dose CSE (10^{-2}–10^{-4} pieces/ml) vs control and low-dose CSE (10^{-5}–10^{-7} pieces/ml) vs control, and combined the data obtained from each experiment. As shown in Fig. 3, contractile sensitivities to OT in the control (n=80) and CSE groups (final concentrations: 10^{-2} pieces/ml, n=18; 10^{-3} pieces/ml, n=27; 10^{-4} pieces/ml, n=20; 10^{-5} pieces/ml, n=18; 10^{-6} pieces/ml, n=27; 10^{-7} pieces/ml, n=31) were 88.5±13.9, 117.2±20.9, 82.6±12.8, 61.5±8.5, 58.3±7.6, 50.8±7.5, and 47.5±6.6 respectively. Sensitivity in the CSE (10^{-7} pieces/ml) group was significantly greater than in the control group (P<0.05). On the other hand, sensitivity in the CSE group with 10^{-2} pieces/ml was significantly lower than that of several of the CSE groups (CSE 10^{-4}–10^{-7} pieces/ml; P<0.05).

Contractile activity (force and frequency) of rat myometrial strips treated with CSE

In the rat CSE (10^{-6} and 10^{-7} pieces/ml) and the control groups, contractile force and frequency were evaluated in rat myometrial strips, which had the same sensitivity to OT (50 μU/ml). Contractile force was expressed as the relative amplitude of high K^+-stimulated contraction. Eight myometrial strips (29.6%) in the rat CSE group with 10^{-6} pieces/ml responded to OT at a concentration of 50 μU/ml, and the relative amplitude was 100.2±6.1%. Eleven myometrial strips (35.5%) from the rat CSE group with 10^{-7} pieces/ml responded to OT at the same concentration, with a relative amplitude of 97.8±4.0%. Fourteen myometrial strips (17.5%) from the control group responded to OT at the same concentration (relative amplitude, 95.3±1.9%). No significant difference was found in the contractile force between the three groups (Fig. 4A). The frequency of OT (50 μU/ml)-induced contraction was 7.8±1.1/10 min (range, 2.8–13) in the rat CSE group with 10^{-6} pieces/ml, 7.9±0.9/10 min (range, 3.5–14.5) in the rat CSE group with 10^{-7} pieces/ml and 6.8±0.6/10 min (range, 4.2–9) in the control group. Low-dose rat CSE groups (CSE 10^{-6} group and especially the CSE 10^{-7} group) tended to

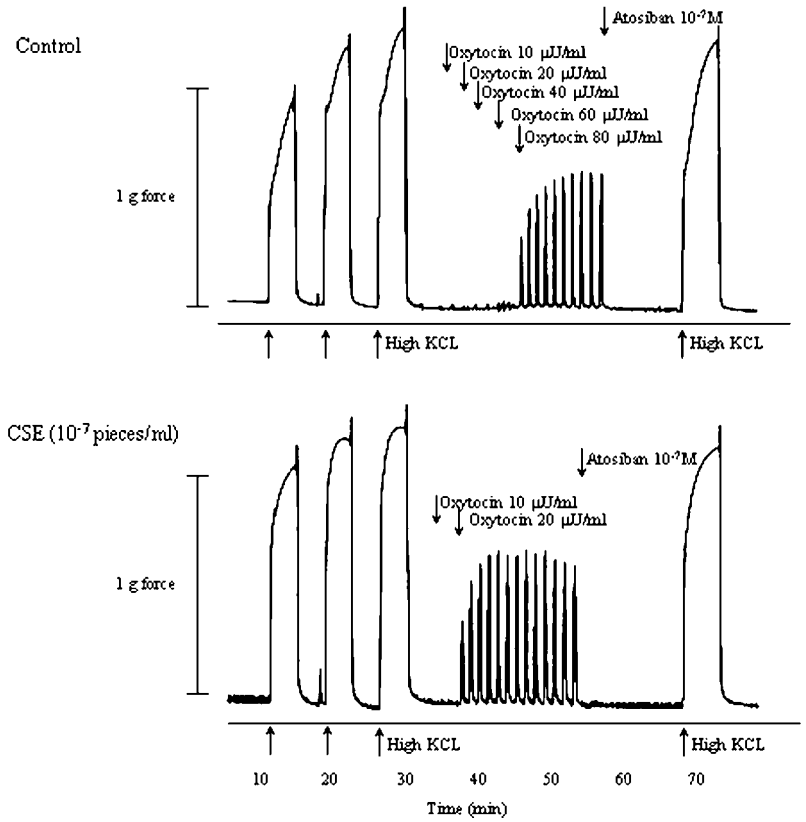

Figure 2 Effect of atosiban on the OT-induced contractions of rat myometrial strips treated with vehicle and CSE 10^{-7} pieces/ml. Contractile sensitivity in control and CSE-treated strips presented here were 80 and 20 μU/ml OT respectively. Both these OT-induced contractions were completely inhibited by atosiban (10^{-7} M).

have higher frequency than the control group, but without significant differences (Fig. 4B).

Expressions of OTR mRNA and FP mRNA in rat myometrial strips treated with CSE

We compared the levels of rat OTR mRNA among rat myometrial strips treated with CSE (10^{-6} and 10^{-7} pieces/ml) and the control groups using quantitative real-time PCR. In one experiment, we used eight strips in each group obtained from one rat. In addition, we repeated the same experiment using another two animals and obtained similar change from another two experiments. However, we did not combine the data, because the expression of rat OTR mRNA in the control group obtained from the three experiments was very varied. Therefore, we presented a typical result obtained from one experiment in which eight strips in each group obtained from one rat were used. The expression levels of rat OTR mRNA in the CSE group with 10^{-7} pieces/ml were significantly greater than the control group (0.0043 ± 0.0010 vs 0.0015 ± 0.0004, $P<0.01$), as shown in Fig. 5A. The expression

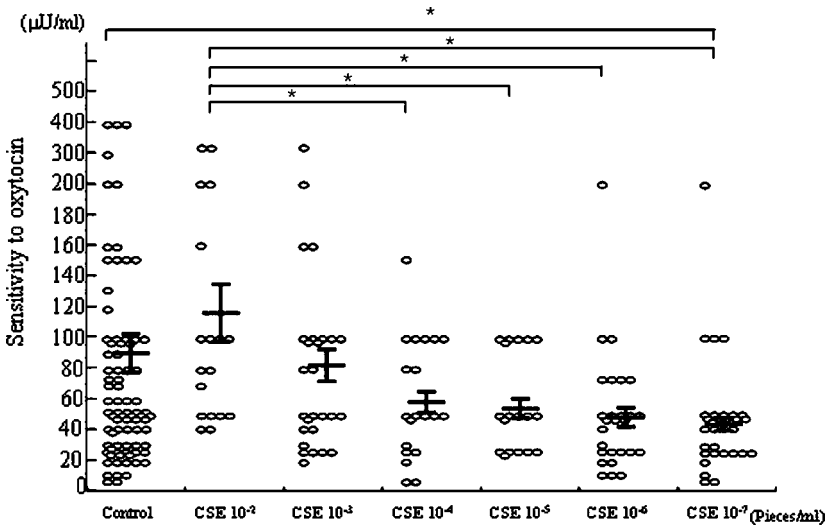

Figure 3 Contractile sensitivity to OT in rat myometrial strips treated with vehicle and several concentrations of CSE. Control ($n=80$) and CSE (10^{-2} pieces/ml ($n=18$), 10^{-3} pieces/ml ($n=27$), 10^{-4} pieces/ml ($n=20$), 10^{-5} pieces/ml ($n=18$), 10^{-6} pieces/ml ($n=27$), 10^{-7} pieces/ml ($n=31$)) groups were used for evaluation of the contractile sensitivity to OT. The range of final concentrations of tested OT was 10–400 μU/ml. Columns and vertical bars represent the mean ± S.E.M. Symbol * shows significant difference ($P<0.05$).

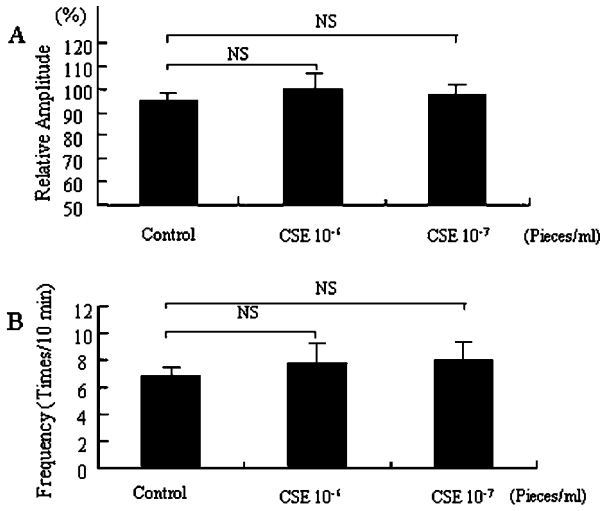

Figure 4 Comparison of contractile activity to 50 μU/ml of OT among control ($n=14$) and CSE (10^{-6} pieces/ml ($n=8$), 10^{-7} pieces/ml ($n=11$)) groups. Uterine contractile activity was evaluated as the relative amplitude and frequency of contractions. (A) Relative amplitude was expressed as the rate of the amplitude of high K^+-induced muscle contraction. (B) The frequency was measured for over 30 min and is expressed as the mean value/10 min.

levels of rat OTR in the CSE group with 10^{-6} pieces/ml (0.0017 ± 0.0004) were slightly higher than the control group, but without significance. There were no significant differences in the expression of rat FP mRNA among the three groups (Fig. 5B).

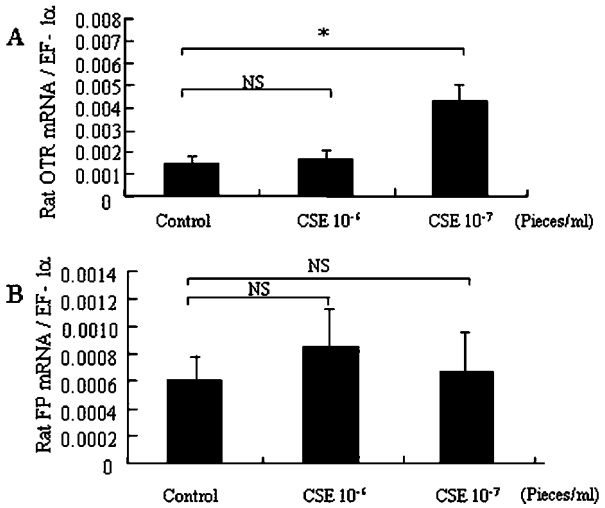

Figure 5 Real-time PCR analysis of the expressions of OTR mRNA and FP mRNA in rat myometrial strips treated with vehicle and CSE. The mRNA expressions levels of OTR and FP were evaluated in comparison with the corresponding EF-1α mRNA from eight strips each of the control and CSE (10^{-6} and 10^{-7} pieces/ml) groups. The final result is expressed as $2^{-\Delta Ct\ (\text{rat OTR or rat FP})}$. Three series of this real-time PCR experiment were performed using a different rat for each series. (A) Comparison of the OTR mRNA expression levels in the control and CSE groups. (B) Comparison of the FP mRNA expression levels in the control and CSE groups. Columns and vertical bars represent the mean ± S.E.M. The symbol *shows significant difference ($P<0.01$).

Expression of OTR mRNA and FP mRNA in human myometrial strips treated with CSE

Based on our results regarding the expression of rat OTR mRNA in myometrium treated with CSE, we examined the levels of OTR mRNA in human myometrium treated with CSE (10^{-7} pieces/ml). In the present study, we employed eight myometrial strips per group, prepared from identical sample tissue, obtained from one preterm patient in one experiment. We also evaluated the expression of human OTR mRNA in the myometrial strip obtained from two preterm patients, in two other experiments respectively, and obtained similar change from another two experiments. However, we did not combine the data, because the expression of human OTR mRNA in control group obtained from the three experiments was very varied. Therefore, we presented a typical result obtained from one experiment in which eight myometrial strips per group obtained from one preterm patient were used (Fig. 6A). The expression levels of OTR mRNA in the preterm group treated with CSE (10^{-7} pieces/ml) were significantly greater than the

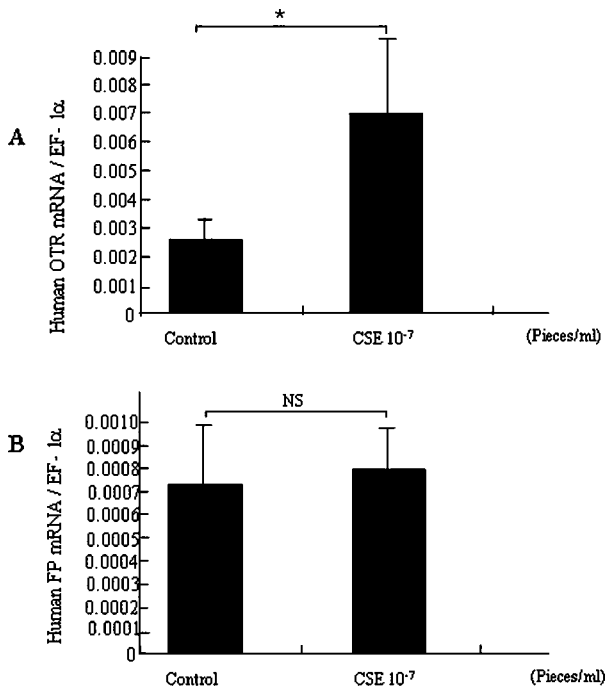

Figure 6 Real-time PCR analysis of the expressions of OTR mRNA and FP mRNA in human preterm myometrial strips treated with vehicle and CSE. The mRNA expression levels of OTR and FP were evaluated in comparison with the corresponding EF-1α mRNA from eight strips each of the control and CSE (10^{-7} pieces/ml) groups. The final result is expressed as $2^{-\Delta Ct\ (\text{human OTR or human FP})}$. Three series of this real-time PCR experiment were performed using myometrial samples obtained from different preterm deliveries for each series. (A) Comparison of the OTR mRNA expression levels in the control and CSE groups. (B) Comparison of the FP mRNA expression levels in the control and CSE groups. Columns and vertical bars represent the mean ± S.E.M. The symbol * shows significant difference in the two groups ($P<0.05$).

control group (0.00675 ± 0.003 vs 0.00251 ± 0.001, $P<0.05$). However, there were no significant differences in the expression of FP mRNA between the preterm CSE groups and the control group (Fig. 6B). In term myometrium, as shown in Fig. 7A and B, expression of both OTR and FP mRNA did not differ significantly between the CSE (10^{-7} pieces/ml) and the control groups. We repeated the same experiments by using sample tissues obtained from another two term patients and found no significant differences in the expression of both OTR and FP mRNA between control and CSE groups.

Contractile sensitivity to OT and PG $F_{2\alpha}$ in human preterm myometrial strips treated with CSE

We employed human myometrium obtained from a woman at 29-week gestation and evaluated contractile sensitivity to OT in myometrial strips treated with low-dose CSE (10^{-7} pieces/ml). As shown in Fig. 8, contractile sensitivity of the myometrial strip treated with CSE (10^{-7} pieces/ml) was higher than the control strip. The myometrial strip treated with CSE contracted rhythmically with OT at a concentration of 300 μU/ml. However, in the control strip, the first contraction was observed at concentrations as high as 3000 μU/ml. Additionally, we detected no rhythmic contractions after the first contraction. After we washed out the myometrial strip and confirmed no effect of OT, we added PG $F_{2\alpha}$ to evaluate whether the sensitivity of the myometrial strip to PG $F_{2\alpha}$ was influenced by CSE. Myometrial strips treated with both CSE and control medium contracted at the same concentration, i.e. 5×10^{-6} M of PG $F_{2\alpha}$.

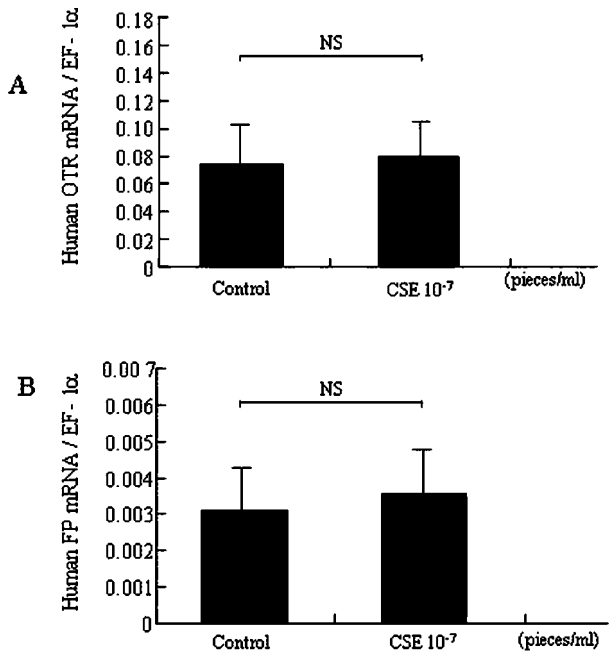

Figure 7 Real-time PCR analysis of OTR mRNA and FP mRNA expression in human term myometrial strips treated with vehicle and CSE. The mRNA expression levels of OTR and FP were evaluated in comparison with the corresponding EF-1α mRNA from eight strips each of the control and CSE (10^{-7} pieces/ml) groups. The final result is expressed as $2^{-\Delta Ct\ (\text{human OTR or human FP})}$. Three series of this real-time PCR experiment were performed using myometrial samples obtained from different term deliveries for each series. (A) Comparison of the OTR mRNA expression levels in term myometrium in the control and CSE groups. (B) Comparison of the FP mRNA expression levels in term myometrium in the control and CSE groups. Columns and vertical bars represent the mean ± S.E.M.

Discussion

Many authors have reported that cigarette smoking during pregnancy causes not only intrauterine growth retardation, but also preterm delivery, and revealed that the risk of preterm delivery among mothers, who smoked during pregnancy, was 1.2–2.6 times higher than among the non-smokers (Hartikainen-Sorri & Sorri 1989, Ferraz *et al.* 1990, De Haas *et al.* 1991, Virji & Cottington 1991, Kramer *et al.* 1992, Cnattingius *et al.* 1993, Wisborg *et al.* 1996). Although the mechanisms through which cigarette smoke cause preterm uterine contractions and preterm delivery have not been known, it is well known that OT and PG $F_{2\alpha}$ induce pregnant uterine contractions and preterm delivery (Takahashi *et al.* 1980, Fuchs *et al.* 1984, Ivanisevic *et al.* 2001). We have recently demonstrated that inhalation of cigarette smoke for 3 days (seven cigarettes/day) by pregnant Wistar rats significantly reduces both fetal and placental weight (Egawa *et al.* 2003), and confirmed that smoking during pregnancy is an important cause of low birth weight, as has often been reported. We have also demonstrated that the sensitivity of pregnant rat uterus to OT (but not PG $F_{2\alpha}$) was significantly increased in the smoking group in comparison with the control group, and the expression of OTR mRNA, but not FP mRNA, in the uterus was significantly increased in the smoking group (Egawa *et al.* 2003). However, it remains unclear whether the inhalation of cigarette smoke directly enhances the expression of OTR mRNA in the myometrium. Since OTR levels are known to be upregulated through progesterone regression, which occurs during luteolysis in the ewe (Hixon & Flint 1987, Zhang *et al.* 1992), the increased expression of OTR mRNA in the pregnant rat myometrium, in the smoking group, may be caused indirectly through progesterone regression via luteolysis. Thus, inhalation of cigarette smoke may damage the function of the corpus luteum in the pregnant rat.

In the present study, we investigated whether cigarette smoke directly influences the contractile sensitivity and activity of the myometrium upon exposure to OT and/or PG $F_{2\alpha}$ using *in vitro* experimental procedures under non-hormonal conditions; we also evaluated the mRNA expression levels of respective receptors. Contractile sensitivity in response to OT in the CSE group treated

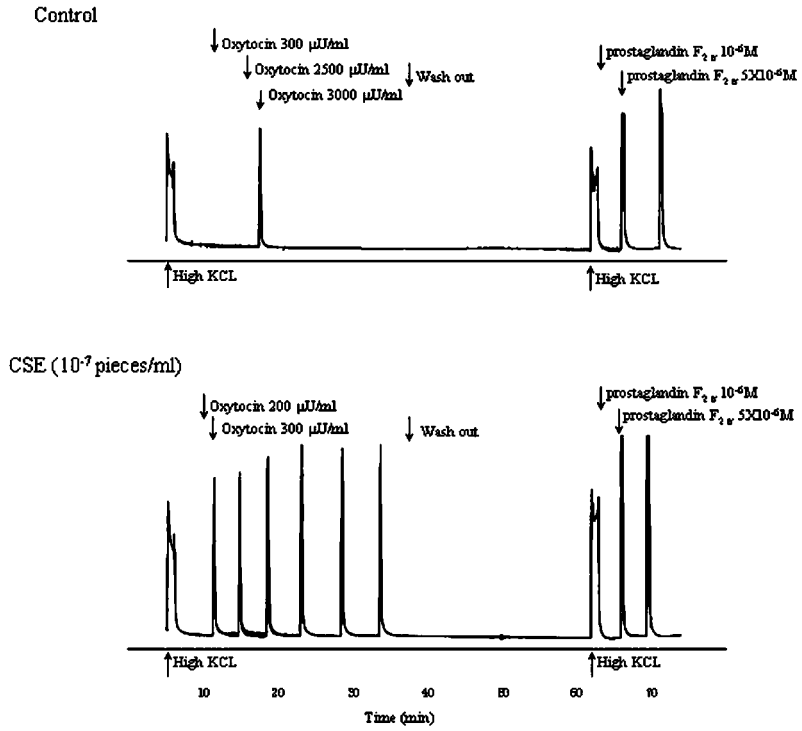

Figure 8 OT and $PGF_{2\alpha}$-induced contractions of human preterm myometrial strips (29 weeks of pregnancy) treated with vehicle or CSE 10^{-7} pieces/ml. Uterine sensitivity was evaluated as the concentration of OT and PG $F_{2\alpha}$, when the first rhythmic myometrial contraction occurred. The myometrial strip with CSE 10^{-7} pieces/ml showed rhythmic contractions at a concentration of 300 μU/ml of OT, but the myometrial strip with vehicle had no rhythmic contractions with the same concentration of OT. After washing out to remove the effect of OT, the same sensitivity to PG $F_{2\alpha}$ (5×10^{-6} M) was found in both the control and CSE (10^{-7} pieces/ml strips).

with a low-concentration CSE (10^{-7} pieces/ml) was significantly greater than in the control group, as shown in Fig. 1–3. The rhythmic contractions found in the control- and CSE-treated strips after the administration of OT were confirmed to be contractions caused via OT-OTR pathway, since they were completely blocked by the administration of atosiban (final concentration, 10^{-7} M), a specific OT antagonist. However, a high-concentration CSE (10^{-2} pieces/ml) was likely to decrease contractile sensitivity in response to OT because of its cytotoxicity, as some myometrial strips were inactive after overnight treatment with CSE (10^{-2} piece/ml). On the other hand, sensitivity in response to PG $F_{2\alpha}$ did not differ significantly between the CSE (10^{-7} pieces/ml) and the control groups (data not shown). These effects were consistent with the effects of inhaled cigarette smoke on contractile sensitivity in response to OT and PG $F_{2\alpha}$ in our previous report (Egawa *et al.* 2003). Among the reasons why low-concentration CSE (10^{-7} pieces/ml) increases contractile sensitivity in response to OT, may be the cytotoxicity of high-concentration CSE, as mentioned above. Another reason may be the relationship between inhibitory and stimulatory substances included in CSE. In high-concentration CSE, the inhibitory effects on contractile sensitivity in response to OT may balance against the stimulatory effect. In low-concentration CSE, the inhibitory effect may be hidden, while the stimulatory effect may be highlighted.

In the present study, both the amplitude and frequency of rhythmic contractions induced by OT did not differ significantly between the CSE (10^{-7} pieces/ml) and the control groups (Fig. 4A and B). However, in our previous study (Egawa *et al.* 2003), the frequency of rhythmic contractions induced by OT was significantly higher in the cigarette smoke group than in the control group, although the amplitude of contractions was similar in both groups. The reason why the inhalation of cigarette smoke, but not CSE, increases the frequency of OT-induced rhythmic contractions is not known. A mechanism by which inhaled cigarette smoke directly or indirectly increases the frequency of OT-induced rhythmic contractions may exist in the regulation of frequency. However, the mechanism by which spontaneous or agonist-induced rhythmic contractions occurs, whereby contraction and relaxation are repeated alternately at suitable intervals for mother or fetus, is still not understood. Further experiments and hypotheses are needed to address this problem.

Our experimental results regarding contractile sensitivity in response to OT suggest that the number of OTRs in uterine myometrium was higher in the low-concentration CSE group than in the control group, and that the number of FPs was similar between the two groups. As expected, real-time PCR revealed that the levels of OTR mRNA expression in day 16 rat myometrium of the CSE group (10^{-7} pieces/ml) were significantly higher than those in the control group (Fig. 5A), while the levels of FP mRNA expression were similar between the two groups (Fig. 5B).

We also examined whether low-concentration CSE enhanced the levels of OTR mRNA expression in pregnant human myometrium, and the results obtained were similar to those observed for rat OTR mRNA expression. The OTR mRNA expression levels in the preterm human myometrium of the CSE group

(10^{-7} pieces/ml) were significantly higher than those in the control group (Fig. 6A). However, the levels of FP mRNA expression in preterm human myometrium were similar between the two groups (Fig. 6B). In term human myometrium, however, the levels of both OTR mRNA and FP mRNA expression did not significantly change between the CSE and control groups (Fig. 7A and B). We observed the same phenomenon in pregnant rat myometrium. On day 21 of pregnancy, the levels of OTR mRNA expression in rat myometrium did not significantly differ between the CSE and control groups (data not shown). One reason why low-concentration CSE increases contractile sensitivity in both human and rat preterm myometrium, but not in term myometrium, may be the basic level of OTR mRNA expression in term myometrium, which is much greater than in preterm myometrium so that the increment of OTR mRNA expression caused by the low-concentration CSE is not highlighted. Another reason may be that OTR mRNA in the myometrium is fully expressed at term so that the response to low-concentration CSE is negligible or lost. Although these findings do not completely explain the mechanism by which preterm delivery is caused by cigarette smoking during pregnancy, they are very important to increase our understanding of this issue.

The experimental results obtained from the studies of OTR mRNA expression in preterm human myometrium suggest that contractile sensitivity in response to OT in preterm human myometrium was higher in the low-concentration CSE group than in the control group, but was similar between the two groups in response to PG $F_{2\alpha}$. As expected, the preterm myometrial strip (29-week gestation) in the low-concentration CSE group responded to 300 μU/ml (final concentration) of OT, and the rhythmic contractions continued until washing out of the medium (Fig. 8). However, such a response was not achieved in the control group until the concentrations reached as high as 3000 μU/ml. Furthermore, rhythmic contractions did not occur even at the high concentration of 3000 μU/ml. On the other hand, as expected, the strips in these two groups responded to the same concentrations of PG $F_{2\alpha}$ (5×10^{-6} M) and the rhythmic contractions continued in the same fashion (Fig. 8).

Many factors influence OTR levels in the pregnant uterus. It is known that steroid hormones (e.g. estrogen, progesterone), inflammatory cytokines (e.g. interleukin-1β, interleukin-6), OT, and lactation suckling influence the expression of OTR at the mRNA or protein level (Phaneuf *et al.* 1997, Phaneuf *et al.* 1998, Fang *et al.* 2000, Rauk & Friebe-Hoffmann 2000, Mitchell & Schmid 2001, Schmid *et al.* 2001, Helmer *et al.* 2002). However, the influence of these factors was limited in the present study using *in vitro* experiments under non-hormonal conditions. It is well known that cigarette smoke contains thousands of chemical compounds. These chemical compounds include alkaloids (nicotine, cotinine, anabasine), carbon monoxide, tar, polyaromatic hydrocarbons, thiocyanate, and many metal ions, including cadmium, lead, chromium, aluminum, and copper. These compounds may directly influence OTR mRNA expression in the uterine myometrium. However, there have been no reports regarding the relationship between these compounds and OTR mRNA expression. Of these compounds, we thought nicotine would be a candidate to upregulate OTR mRNA, because nicotine has many pharmacological and physiological activities. Therefore, we employed α-bungarotoxin, an antagonist of nicotine, to investigate whether nicotine contained in CSE was involved in the increased OTR mRNA. However, α-bungarotoxin did not inhibit the effect of CSE on OTR mRNA expression in preterm myometrium (data not shown). We consider other possibilities except OTR in the elevated response to OT in CSE group. It has been reported that protein kinase c (PKC) activation induces vascular and tracheal smooth muscle contractions by increasing the Ca^{2+} sensitivity of contractile elements. Additionally, PKC has been reported to play an important role in the OT-induced smooth muscle contractions. Therefore, we investigated conventional PKC mRNA expression (PKCα, -β, -γ) in the control and CSE groups. However, there was no significant difference in the conventional PKC mRNA expression between two groups (data not shown). Further studies are needed to examine the mechanism by which CSE directly increases the levels of OTR mRNA expression in preterm myometrium.

In summary, our results from *in vitro* experiments under non-hormonal conditions indicate that CSE directly increases the contractile sensitivity of rat and human preterm myometrium in response to OT through a mechanism involving the stimulation of OTR mRNA expression. These findings suggest that smoking during pregnancy increases the risk of preterm labor and delivery by increasing the level of myometrial OTRs. Further studies on compounds that upregulate the levels of OTR mRNA in the myometrium may yield valuable information regarding the physiology and pathophysiology in parturition.

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