Corticotropin-releasing hormone activates connexin 43 via activator protein-1 transcription factor in human myometrial smooth muscle cells

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Wu X, Shen H, Yu L, Peng M, Lai WS, Ding YL. Corticotropin-releasing hormone activates connexin 43 via activator protein-1 transcription factor in human myometrial smooth muscle cells. Am J Physiol Endocrinol Metab 293: E1789–E1794, 2007. First published September 25, 2007; doi:10.1152/ajpendo.00249.2007.—Corticotropin-releasing hormone (CRH) and connexin 43 (Cx43) play crucial roles in uterine contraction and the onset of labor. The aim of the present study was to investigate the regulatory effects of CRH on Cx43 expression in human myometrial smooth muscle cells (SMCs) and, potentially, its activation of the c-Fos/activator protein (AP)-1 signaling pathway. Human myometrial SMCs collected from nonpregnant women were treated with different concentrations of CRH. Transient transfection of AP-1 decoy oligodeoxynucleotide (ODN) was used to block AP-1 sites of Cx43. The transcriptional activity of AP-1 was detected by luciferase assay. Cx43 protein expression was visualized by immunofluorescence staining. mRNA and protein expression of Cx43 and Cx43 were demonstrated by real-time quantitative RT-PCR and Western blot, respectively. CRH facilitated Cx43 expression and enhanced AP-1 promoter activity in human uterine SMCs. After CRH treatment, Cx43 expression in the cytoplasm increased significantly. CRH significantly increased mRNA and protein expression of c-Fos and Cx43 in a dose-dependent manner (P < 0.01). A transient transfection of AP-1 decoy ODN did not affect CRH regulation of c-Fos (P > 0.05) but almost completely abolished CRH-induced enhancement of Cx43 expression (P < 0.01). In human primary myometrial SMCs, CRH enhances Cx43 mRNA and protein expression through upregulation of c-Fos expression. Blockade of AP-1 sites to the Cx43 promoter can neutralize the CRH-induced upregulation of Cx43.

c-Fos/activator protein-1; human myometrial smooth muscle cells; onset of labor

Premature and postterm births are major causes of neonatal morbidity and mortality. However, the onset of labor in humans is a complicated process that has not been fully clarified. Understanding the complex physiological process and the mechanism of the onset of labor may provide novel therapeutic strategies to prevent and treat premature delivery or postterm pregnancy.

Corticotropin-releasing hormone (CRH), a 41-amino acid peptide, has been demonstrated to play an important role in uterine contraction and onset of labor (9, 12). In the nonpregnant state, CRH is mainly released by the hypothalamus and remains at a low level. During pregnancy, the placenta also synthesizes CRH and gradually becomes the major source of CRH in the human body (5). CRH concentration in peripheral blood is very low in the first trimester of pregnancy. Because of the large amount of CRH synthesized by the placenta beginning in the second trimester of pregnancy, plasma CRH increases exponentially with time (weeks) of gestation. Then it increases significantly and reaches its peak (585 pmol/l) at the onset of labor. CRH falls to its normal level (0.2–2 pmol/l) 24 h after delivery (19). Therefore, CRH is generally considered a promotor of labor (9, 12), but the labor-promoting mechanism of CRH remains unclear.

The increased expression of connexin 43 (Cx43) in uterine muscle is an important factor in uterine contraction and onset of labor (10, 15). In the nonpregnant state and the first trimester of pregnancy, Cx43 expression in the myometrium is minimal. However, Cx43 mRNA and protein levels increase dramatically before labor (18). The nuclear transcription factor activator protein (AP)-1 can combine with many genes and, subsequently, regulate transcription of these target genes. There are AP-1 sites in the Cx43 gene promoter region. AP-1 facilitation of the transcription and expression of Cx43 in myometrial cells has been documented by Wu et al. (28) and Mitchell and Lye (14). Mitchell and Lye proved that c-Fos, the AP-1 subunit, plays an important regulatory function in Cx43 expression. Becquet et al. (3) and Autelitano and Cohen (2) found that CRH could facilitate AP-1 and upregulate expression of its subunit c-Fos in AtT-20 cells. However, no information about the association between the level of CRH and the change in c-Fos/AP-1 in myometrial tissues is available in the literature. Moreover, the mutual regulation among CRH, Cx43, and the AP-1 subunit c-Fos in the mechanism of labor onset remains unclear.

We hypothesize that CRH may influence Cx43 expression in human myometrial smooth muscle cells (SMCs) through AP-1 and, thus, initiate labor. To investigate the regulatory effects of CRH on Cx43 expression in human myometrial cells and its potential activation of the c-Fos/AP-1 pathway, we employed primary cultured myometrial SMCs and a specific block of AP-1 sites.

Materials and Methods

Human samples and myometrial SMCs. Human uterine tissue (1.0 × 1.0 × 1.0 cm3) was collected from six patients who had undergone hysterectomy for cervical intraepithelial neoplasia. The mean parity of these six nonpregnant women (27–40 yr of age) was 1.3. All the patients were menstruating, and none was taking medication. The study was approved by the Research Ethics Committee of the Central South University of China. Informed consent for the use of tissue was obtained from each patient.

Fresh tissue was added to DMEM containing 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA). Primary
human myometrial SMCs were dispersed using the collagenase II (1 mg/ml) digestion method and DNase (0.15 mg/ml; Invitrogen) and subsequently purified by a discontinuous Percoll density gradient (1.03–1.06), as described elsewhere (6). Human myometrial SMCs were harvested, washed, and plated on uncoated plastic tissue culture flasks at 25,000 cells/cm². Myocyte viability was >93% as determined by trypan blue staining. The purity of myometrial SMCs, which were identified by the typical fusiform-like configuration under a light microscope, immunocytochemical staining with antibody against smooth muscle α-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and Western blot detection of h-calponin, h-caldesmon, and smooth muscle α-actin, was always >92% (8). The primary cell cultures of freshly isolated uterine SMCs were initiated in DMEM supplemented with 15% FBS, antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin), and 2 mM l-glutamine at 37°C in a humidified atmosphere of 5% CO₂. Thereafter, the human myometrial SMCs at passage 3 were transferred into 22 cells were fixed with methanol for 3 min at 20°C, washed twice with cold PBS, and blocked with 200 μl of 1% normal goat serum at 37°C for 45 min. They were then incubated with polyclonal rabbit antibody against human Cx43 (Santa Cruz Biotechnology; 1:50 dilution) at 37°C for 1 h. After they were washed, the cells were covered with the Cy3-conjugated secondary antibody goat anti-rabbit IgG (Chemicon, Temecula, CA; 1:50 dilution) and incubated at 37°C for 1 h. Then 4,6-diamidino-2-phenylindole (0.1 mg/ml in 0.1 M PBS) was used to stain the nuclei at room temperature for 20 min. The Cx43-stained cells were visualized using an Olympus fluorescence microscope.

Transcript transfection and luciferase assay. To address the effects of CRH on the transcriptional activity of AP-1, human myometrial SMCs at passage 3 in serum- and antibiotic-free DMEM were cotransfected with 1.03 ng of the luciferase (Luc) reporter plasmid pAP-1-Luc (kindly provided by Prof. Xiushan Wu, College of Life Sciences, Hunan Normal University) and 0.1 μg of the β-galactosidase (β-Gal) plasmid pSV-β-Gal (Promega, Madison, WI) with use of Lipofectamine 2000 reagent (Invitrogen). After 36 h of transfection, CRH was added to the culture medium at 5.85, 58.5, 585, and 5,850 pmol/l. Total cellular protein and total AP-1 decoy ODN transfection, the cells were treated for 8 h with CRH at 5.85, 58.5, 585, and 5,850 pmol/l. Total cellular protein and total RNA were extracted for subsequent analysis.

Real-time RT-PCR quantitation of human c-Fos and Cx43 mRNA. Total RNA was extracted from cells treated for 8 h with CRH by TRIzol reagent according to the manufacturer’s instructions. Cx43 mRNA was quantified by Light Cycler according to the real-time RT-PCR protocol provided by Roche Molecular Biochemicals (Mannheim, Germany). Briefly, full-length human c-Fos and Cx43 cDNA were inserted into a pCR2.1 vector (Invitrogen). The sense mRNA of the target gene was synthesized by the Riboprobe Combination System T3/T7 kit (Promega) with T7 polymerase. The in vitro transcribed sense mRNA of c-Fos and Cx43 were used as the respective templates for the standard curve. For real-time RT-PCR quantitation of c-Fos and Cx43, 1 μg of total RNA from each sample was employed (20). The sense primer sequence of c-Fos (GenBank accession no. M16287) was 5’-CTG GGG GTC GGC ACT TGC T-3’ (position at 1167–1189), and the antisense primer sequence was 5’-GGA GAT TGC CGC TTT CTG CCA CCT-3’ (position at 1353–1377); the expected size of the product was 210 bp. The sense primer sequence of Cx43 (GenBank accession no. NM_000165) was 5’-TTG CTG CTG ACC AAC ATC ATC GCA CCT-3’ (position at 658−682), and the antisense primer sequence was 5’-GCC AGG GAC ACC AAC GAC AC-3’ (position at 853−873); the expected size of the product was 215 bp. Real-time RT-PCR was performed with the Light Cycler-RNA Master SYBR Green 1 kit (Roche Molecular Biochemicals) as follows: denaturation at 95°C for 2 min, 40 cycles of amplification for 1 s at 94°C, 10 s at 58°C, and 16 s at 72°C. Fluorescence intensity was determined at 58°C for 10 s at the end of each cycle. The amount of c-Fos and Cx43 mRNA was calculated and expressed as nanograms per milligram of standard RNA.

Protein isolation and Western blot analysis of c-Fos and Cx43 protein expression. CRH-treated cells were washed twice with cold PBS and then lysed in cellular protein extraction solution [1× = 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l EDTA (pH 8.0), 10 mmol/l NaCl, 1% SDS, 1 mmol/l PMSF, and 0.25 mol/l sucrose], as described previously (22). Protein concentration was measured by the Lowry method. Protein (25 μg) was separated on a 10% SDS-polyacrylamide gel under reducing conditions and transferred onto a Nitroplus-2000 membrane (Membrane Separations, Westbrook, MA). Nonspecific antibody binding was blocked by preincubation of the membranes in 1× Tris-buffered saline containing 5% skim milk for 1 h at room temperature. Membranes were then incubated overnight at 4°C with the antibody against c-Fos and Cx43 (Santa Cruz Biotechnology; 1:500 dilutions). After they were washed, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ; 1:1,000 dilution) for 1 h at room temperature. Bands were visualized using the ECL kit (Amersham Biosciences) according to the manufacturer’s instructions (21). β-Anti protein expression was used as an internal control in this Western blot procedure. The protein band intensities were measured with an image analysis program (IMAGE J, National Institutes of Health, Bethesda, MD).

Statistical analyses. Values are means ± SD. Statistical significance of differences was determined using a one-way ANOVA followed by Tukey’s post test with SPSS software (version 13.0, SPSS, Chicago, IL). Differences were considered to be significant at P < 0.05.
RESULTS

**CRH-facilitated Cx43 expression in human myometrial SMCs.** In the control non-CRH-treated group, Cx43 was not expressed in human myometrial SMCs. After the cells were treated with CRH for 8 h, Cx43 was expressed in the cytoplasm. Cx43 expression was strengthened in a dose-dependent manner as CRH concentration increased from 5.85 to 5,850 pmol/l (Fig. 1).

**CRH-enhanced AP-1 promoter activity in human uterine SMCs.** CRH enhanced AP-1 promoter activity in human myometrial SMCs (Fig. 2). After 36 h of transfection with pAP-1-Luc, myometrial SMCs were treated for 8 h with different concentrations of CRH. The relative AP-1 promoter activity, represented by the ratio of luciferase fluorescence to β-Gal concentration, in the control non-pAP-1-Luc-transfected myometrial SMCs was 850 ± 64. The relative AP-1 promoter activities of the myometrial SMCs transfected with pAP-1-Luc and treated with CRH at 0, 5.85, 58.5, 585, and 5,850 pmol/l were 26,288 ± 6,547, 71,788 ± 8,565, 79,729 ± 9,763, 95,013 ± 15,143, and 258,002 ± 17,512, respectively (Fig. 2). AP-1 promoter activities were significantly different among the groups (P < 0.05), but not between those treated with CRH at 5.85 and 58.5 pmol/l (P > 0.05). AP-1 promoter activities were significantly higher (P < 0.01) in the five experimental groups transfected with pAP-1-Luc than in the control non-pAP-1-Luc-transfected myometrial SMCs. CRH significantly increased AP-1 promoter activity of myometrial SMCs in a dose-dependent manner.

**Influence of CRH on mRNA expression of c-Fos and Cx43 in AP-1 decoy ODN-transfected or nontransfected human SMCs.** Compared with the control non-CRH-treated (0 pmol/l) SMCs, CRH at 5.85, 58.5, 585, and 5,850 pmol/l significantly (P < 0.01) increased c-Fos mRNA expression 2.03-, 5.60-, 9.41-, and 10.19-fold, respectively, in AP-1 decoy ODN-transfected human myometrial SMCs and significantly (P < 0.01) increased Cx43 mRNA expression 2.03-, 5.60-, 9.41-, and 10.98-fold, respectively, in non-AP-1 decoy ODN-transfected human myometrial SMCs and significantly (P < 0.01) increased c-Fos mRNA expression 2.03-, 5.60-, 9.41-, and 10.19-fold, respectively, in AP-1 decoy ODN-transfected human myometrial SMCs (Fig. 3). No significant difference (P > 0.05) was observed between the groups with or without AP-1 decoy ODN transfection with respect to CRH treatment at the same concentration (Fig. 3). CRH enhanced human c-Fos mRNA expression in myometrial SMCs in a dose-dependent manner, but AP-1 decoy ODN did not affect the CRH-induced upregulation of c-Fos mRNA.

![Fig. 1. Regulation of connexin 43 (Cx43) expression and location by corticotropin-releasing hormone (CRH). Cell nucleus was stained with 4,6-diamidino-2-phenylindole, and Cx43 was stained with Cy3. Human myometrial smooth muscle cells (SMCs) were left untreated (A) or treated with CRH at 5.85 (B), 58.5 (C), 585 (D), and 5,850 pmol/l (E).](image1)

**Fig. 2. CRH-induced increase of activator protein (AP)-1 promoter activity in human myometrial SMCs.** –, non-pAP-1-Luc-transfected SMCs; +, pAP-1-Luc-transfected SMCs. Luciferase activity was normalized to β-galactosidase (β-Gal) activity and expressed as luminescence per β-Gal unit per milligram or milliliter of protein. Each experiment was independently repeated 5 times. F = 486.902 for 1-way ANOVA (P < 0.001). Tukey’s post test showed a significant difference between groups (P < 0.05), except between 5.85 and 58.5 pmol/l (P > 0.05). AP-1 promoter activity was significantly higher in pAP-1-Luc- than in non-pAP-1-Luc-transfected SMCs (P < 0.01, respectively).
Compared with control non-CRH-treated (0 pmol/l) SMCs, CRH at 5.85, 58.5, 585, and 5,850 pmol/l significantly (\(P < 0.01\)) increased Cx43 mRNA levels 2.21-, 2.59-, 4.89-, and 12.52-fold, respectively, in human myometrial SMCs without c-Fos/AP-1 binding competition by AP-1 decoy ODN (Fig. 4). After transfection of AP-1 decoy ODN against c-Fos/AP-1 sites, CRH at different concentrations did not change Cx43 mRNA expression in myometrial SMCs (Fig. 4; \(P > 0.05\)). Compared with non-AP-1 decoy ODN-transfected human myometrial SMCs, AP-1 decoy ODN-transfected cells had a significantly decreased Cx43 mRNA level while they were treated with CRH at the same concentration (\(P < 0.01\)). Transfection with AP-1 decoy ODN with the ability to block c-Fos/AP-1 sites significantly abolished the CRH-induced up-regulation of Cx43 mRNA levels in human myometrial SMCs.

**Influence of CRH on protein expression of c-Fos and Cx43 in human SMCs with or without AP-1 decoy ODN transfection.** CRH regulation of protein expression of c-Fos and Cx43 in human myometrial SMCs is demonstrated in Fig. 5. The control non-AP-1 decoy ODN-transfected cells and those transfected with AP-1 decoy ODN were treated with CRH for 8 h, and the protein expression of c-Fos and Cx43 was detected by Western blot. In the cells without AP-1 decoy ODN transfection against AP-1 sites, CRH significantly increased the protein expression of c-Fos and Cx43 in a dose-dependent manner. After transfection with AP-1 decoy ODN to compete with c-Fos/AP-1 binding, the effects of CRH on enhancement of c-Fos expression were the same as those observed in the control myometrial SMCs without AP-1 site competition. However, with AP-1 decoy ODN transfection, CRH lost its Cx43-enhancing ability and even completely blocked Cx43 protein expression.

**DISCUSSION**

CRH is the major regulatory factor in the stress response (26). It has been reported that plasma CRH concentration is very low in the first trimester of pregnancy. The placenta synthesizes large quantities of CRH after the second trimester of pregnancy, and thus plasma CRH increases exponentially...
with time (weeks) of gestation. Finally, it reaches the threshold of labor induction. CRH reaches its peak during labor and declines to its prepregnancy normal level ~24 h after delivery (19). The present data indicate that CRH regulates Cx43 expression in myometrial tissues.

Cx43 plays an important regulatory role in many physiological processes, such as cellular metabolism, proliferation, and differentiation and concordance of smooth muscle contraction (16, 24). In uterine smooth muscle tissues in the nonpregnant state and the first trimester of pregnancy, Cx43 level is relatively low. Cx43 level increases rapidly during the end stage of pregnancy. Until labor, Cx43 molecule not only increases in quantity, but also gets an enlarged volume. Cx43 disappears 24 h after delivery (25). The same changes can be found before delivery. When normal labor starts, the uterine muscle contraction is highly concordant. The stimulation signals are transmitted through the firmly contracted SMCs and quickly to the whole uterus, which is the foundation for the onset of labor. The cell-to-cell communication mediated through gap junctions is essential to the synchronization of contractions in uterine muscle. This special signal conduction is completed through Cx43, which is specifically expressed in human myometrial SMCs (16, 24). Cx43, which forms intercellular channels in the cytoplasmic membrane and induces a synchronous action by the cells with the same biological function, is critically important in junctional communication. The principal process leading to assembly of gap junctions involves the cotranslational insertion of connexin proteins into the endoplasmic reticulum followed by their rapid oligomeric association into homo- or heteromeric connexons, which are transported via the Golgi apparatus to the plasma membrane. Oligomerization is a high-fidelity process that determines connexon channel stoichiometry and conductance characteristics (11).

c-Fos, the subunit of AP-1 that is a kind of nuclear transcription factor commonly seen in organisms, controls the transcription of genes containing AP-1 sites. AP-1 is a dimer composed of different members of the Fos and Jun families. It has been shown that CRH can activate AP-1 and upregulate the expression of its subunit c-Fos (2, 3). Our study indicates that primary myometrical SMCs treated with CRH exhibit a dramatic increase of c-Fos protein expression followed by an increase of Cx43 protein expression in a CRH-dose-dependent manner. The results of pAP-1-Luc transient transfection and luciferase reporter gene assays suggest that CRH stimulates AP-1 promoter activity in human myometrial SMCs. This presumption and the extremely important role of AP-1 in myometrial SMCs. The expression of c-Fos was reported to be associated with Cx43 expression, and this association was regulated by estrogen and progesterone (17). Importantly, our in vitro data indicate that c-Fos is a particularly important upstream mediator of Cx43 expression in human myometrial SMCs. We speculate that only when CRH reaches its threshold at labor does it have the ability to interfere with the dynamic balance of AP-1 regulation in the human body and work as a trigger to promote the abrupt and extreme increase of Cx43 expression required for myometrial “activation” and the onset of labor. This presumption and the extremely important role of AP-1 in the biological signal transduction of Cx43 regulation emphasize the necessity for continuous monitoring of AP-1 levels in pregnant myometrium.

Along with previous findings of other groups regarding the respective effects of CRH and Cx43 on the onset of labor, our data suggest a regulatory chain of CRH in myometrial tissue as follows. When CRH concentration reaches the threshold to induce labor, CRH combines with the specific membrane receptors to trigger the signal transduction leading to the activation of AP-1 (4). AP-1 subsequently combines with the AP-1 sites in the Cx43 promoter region and activates the Cx43 promoter (14, 17). The significant upregulation of Cx43 mRNA and protein contributes to the formation of myometrial gap junction channels. The open gap junctions then strengthen electrical and cellular coupling, provide low-resistance sites, and enable transmission of electrical impulses to the whole myometrium, thus inducing the onset of labor and facilitating synchronization of contraction of uterine muscles in labor (27). This mechanism and the crucial role of c-Fos in this regulatory chain of CRH provide an opportunity to initiate labor at the appropriate time by controlling c-Fos levels in human myometrial SMCs. This possibility is attractive and clinically significant, because it may provide a potential strategy for prevention of preterm labor and treatment of postterm pregnancy, especially for a potential clinical approach to postpone the onset of labor for prevention of preterm delivery by inhibiting AP-1 activation and expression or blocking its binding to the Cx43 promoter in myometrial SMCs. However, this interesting clinical strategy requires further exploration in vivo.

In conclusion, CRH enhances Cx43 mRNA and protein expression through an upregulation of c-Fos expression. Blockade of AP-1 sites of the Cx43 promoter can neutralize the upregulatory effects of CRH on Cx43 in human primary myometrial SMCs.
REFERENCES


