Fasudil Inhibits the Proliferation and Contractility and Induces Cell Cycle Arrest and Apoptosis of Human Endometriotic Stromal Cells: A Promising Agent for the Treatment of Endometriosis

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**Context:** During the development of endometriotic lesions, excess fibrosis may lead to scarring and to the alterations of tissue function that are the characteristic features of this disease. Enhanced extracellular matrix contractility of endometriotic stromal cells (ECSC) mediated by the mevalonate-Ras homology (Rho)/Rho-associated coiled-coil-forming protein kinase (ROCK) pathway has been shown to contribute to the pathogenesis of endometriosis.

**Design:** To assess the use of fasudil, a selective ROCK inhibitor, for the medical treatment of endometriosis-associated fibrosis, the effects of this agent on the cell proliferation, apoptosis, cell cycle, morphology, cell density, and contractility of ECSC were investigated. The effects of fasudil on the expression of contractility-related, apoptosis-related, and cell cycle-related molecules in ECSC were also evaluated.

**Results:** Fasudil significantly inhibited the proliferation and contractility of ECSC and induced the cell cycle arrest in the G2/M phase and apoptosis of these cells. Morphological observation revealed the suppression of ECSC attachment to collagen fibers and decrease of cell density by fasudil. The expression of α-smooth muscle actin, RhoA, ROCK-I, and ROCK-II proteins was inhibited by fasudil administration. The expression of the antiapoptotic factors, Bcl-2 and Bcl-XL, in two-dimensional cultured ECSC were down-regulated by the addition of fasudil, whereas, the expression of p16INK4a and p21Waf1/Cip1 was up-regulated by the addition of fasudil.

**Conclusions:** The present findings suggest that fasudil is a promising agent for the treatment of endometriosis. The inhibition of cell proliferation, contractility, and myofibroblastic differentiation, the attenuation of attachment to collagen fibers, the decrease of cell density, and the induction of cell cycle arrest and apoptosis of ECSC are involved in the active mechanisms of fasudil. (J Clin Endocrinol Metab 96: E1944–E1952, 2011)
myofibroblasts. It has been suggested that type I collagen is a major contributor to endometriosis-associated fibrosis (3, 5). One approach to understanding the pathogenesis of endometriosis is to investigate the mechanisms underlying the fibrogenesis associated with this disease.

We have established a three-dimensional (3-D) collagen gel culture system with human endometriotic stromal cells (ECSC) as a model of fibrosis formation in endometriosis (6–9). In this system, ECSC are cultured in floating collagen lattices to induce the reorganization and compaction of collagen fibers, resulting in the contraction of collagen gels. This culture system provides a model of mechanically relaxed tissue with low tensile strength comparable to that in the early developmental stages of endometriotic lesions. Research on endometriotic stromal cell biology in 3-D collagen matrices offers new opportunities to gain a better understanding of the reciprocal and adaptive interactions that take place between cells and the surrounding matrix in a tissue-like environment. Such interactions are integrated with the regulation of endometriotic tissue morphogenesis and the dynamics that characterize endometriosis-associated fibrosis. Using this model, we demonstrated that ECSC cultured in floating 3-D collagen gel exhibit a more enhanced contractile profile and greater ability to differentiate into a myofibroblastic phenotype than do normal endometrial stromal cells (6–9). Activation of the mevalonate-Ras homology (Rho)/Rho-associated coiled-coil-forming protein kinase (ROCK)-mediated pathway (Fig. 1) in ECSC may be involved in this phenomenon (6–10).

Therefore, it has been suggested that promising drugs for the treatment of endometriosis may eventually be derived from agents that suppress fibrosis formation by targeting the Rho-ROCK-mediated pathway as well as myofibroblastic differentiation. The pyridine derivative Y-27632, the isoquinoline sulfonamide derivative fasudil [hexahydro-1-(5-isoquinolinesulfonyl)-1H-1,4-diazepine hydrochloride, HA1077], and its active metabolite hydroxyfasudil have recently been shown to be selective inhibitors of ROCK (11). We have previously demonstrated that Y-27632 attenuated the contractility of ECSC (6).

Fasudil (Fig. 1) has been marketed since 1995 in Japan for the treatment of ischemic stroke (12, 13), cerebral vasospasm (14), and subarachnoid hemorrhage (15). Fasudil and hydroxyfasudil selectively inhibit ROCK by competing with ATP for binding to the kinase and show minimal effects on other intracellular signaling pathways, such as myosin light chain kinase, protein kinase A, protein kinase C, or protein kinase G (11, 16, 17). In the present study, we investigated the effects of fasudil on the proliferation, apoptosis, and contractility of ECSC. We also discuss a novel therapeutic strategy for endometriosis-associated fibrosis.

Materials and Methods

ECSC isolation procedure and cell culture conditions

Endometriotic tissues were obtained from premenopausal patients in the mid-to-late proliferative phase who had undergone salpingo-oophorectomy or cystectomy for ovarian endometriotic cysts (n = 8, aged 26–34 yr). None of the patients had undergone any hormonal treatments for at least 12 months before the operation. This study was approved by the Institutional Review Board of the Faculty of Medicine at Oita University. ECSC were isolated from ovarian endometriotic tissues by enzymatic digestion as previously described (18). Isolated ECSC were used for the following experiments (18). Each experiment was performed in triplicate and repeated at least four times.

Modified methylthiazoletetrazolium assay

The rate of proliferation of ECSC after fasudil treatment was determined in 96-well plates by a modified methylthiazoletetrazolium assay system using WST-1 (Roche Diagnostics GmbH,
Penzberg, Germany) according to the manufacturer’s protocol. Then, 5 × 10⁴ ECSC in DMEM supplemented with 1% BSA (Sigma-Aldrich, St. Louis, MO) were distributed into each well of a 96-well flat-bottomed microplate (Corning, New York, NY), which was then incubated overnight. The medium was then removed, and the cells were incubated for 48 h with 200 μl experimental medium containing various concentrations of fasudil (0.1–100 μM) (BIOMOL International, L.P., Plymouth Meeting, PA). Thereafter, 20 μl WST-1 dye was added to each well. The wells were then incubated for an additional 4 h. Cell proliferation was evaluated by measuring the light absorbance at 540 nm. Data were calculated as the ratio of values obtained for the treated cells and for the untreated controls.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay

The effects of fasudil on the DNA synthesis of ECSC were also determined by BrdU incorporation using cell proliferation ELISA (Roche Diagnostics). ECSC (1 × 10⁴) in DMEM supplemented with 0.1% BSA were distributed into each well of a 96-well flat-bottomed microplate. After overnight incubation, the medium was removed and the cells were incubated for another 48 h with 100 μl experimental medium containing various concentrations of fasudil (0.1–100 μM). Thereafter, 10 μl BrdU (10 mM) was added to each well, and the plates were further incubated for 2 h. BrdU incorporation was then evaluated by measuring the light absorbance at 450 nm according to the manufacturer’s protocols. Data were calculated as the ratio of values obtained for the treated cells and those for the untreated controls.

Assessment of fasudil-induced cell cycle arrest by flow cytometry

The effects of fasudil on the cell cycle of ECSC were analyzed by flow cytometry, as previously described (19). Briefly, ECSC were cultured at less than 60% confluence for 48 h with or without the presence of fasudil (100 μM). They were then trypsinized, washed in PBS, fixed in 70% methanol, and incubated for 120 min at 4°C in the dark with a solution of 5 μg/ml propidium iodide (Sigma-Aldrich), and 0.1% Nonidet P-40 (Sigma-Aldrich). Flow cytometric analysis of the cell cycle was performed after propidium iodide staining using the CellFIT program (Becton Dickinson, Sunnyvale, CA), in which the S-phase was calculated using a ModFit model.

Assessment of fasudil-induced apoptosis by a cell death detection ELISA

Fasudil-induced apoptosis of ECSC was quantified by direct determination of nucleosomal DNA fragmentation by cell death detection ELISA (Roche Diagnostics) as previously described (19). The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Briefly, 1 × 10⁶ cells were plated on 24-well culture plates (Corning) and cultured overnight. The supernatant was replaced with fresh culture medium containing various amounts of fasudil (0.1–100 μM). Forty-eight hours after stimulation, the cells were lysed according to the manufacturer’s instructions, followed by centrifugation (200 × g for 5 min). The mono- and oligonucleosomes contained in the supernatants were determined using an anti-histone-biotin antibody. The concentration of nucleosomes-antibody pairs was determined photometrically at a wavelength of 405 nm using 2,2'-azino-di(3-ethylbenzothiazoline-sulfonate) as a substrate. Data were calculated as the ratio of the values obtained for the treated cells and for the untreated controls.

Collagen gel contraction assay

To assess the effects of fasudil on the contractility of ECSC, cellular collagen gel contraction assays were performed as previously described (6, 8, 20). A sterile solution of acid-soluble collagen type I purified from porcine tendons (Cellmatrix type I-A; Nitta Gelatin Inc., Osaka, Japan) was prepared according to the manufacturer’s instructions. ECSC were embedded in collagen gel and cultured three-dimensionally. Briefly, the ECSC were suspended in collagen solution (3.0 × 10⁵ cells/ml), and this collagen/cell mixture (2 ml/plate) was dispensed into 35-mm culture plates (Corning) coated with 0.2% BSA, after which the mixture was allowed to polymerize at 37°C for 30 min. Immediately after polymerization, 1 ml culture medium containing fasudil (final concentration, 0.1-100 μM) was added to each plate. After incubation for 48 h, the collagen gels were photographed, and the area of the gel surface was measured with the public domain image program ImageJ version 1.44 developed at the National Institutes of Health (Bethesda, MD).

Morphology and cell density of ECSC in 3-D collagen gel culture

Upon termination of the collagen gel contraction assay, the contracted collagen gels treated with or without fasudil (100 μM) were fixed with 4% paraformaldehyde, stained with Giemsa solution, and dried overnight on the slide glass. The morphological features and the density of the cells were observed by light microscopy.

Western blot analysis

The expression of α-SMA, Rho A, ROCK-I, and ROCK-II in ECSC in the above-mentioned 3-D culture and Bcl-2, Bcl-XL, p16INK4a, and p21Waf1/Cip1 in the conventional 2-D culture were investigated by Western blotting analysis. ECSC in the 3-D and 2-D cultures were treated with fasudil (100 μM) for 48 h. The contracted collagen gels were minced and incubated in 0.02% collagenase type I (Sigma-Aldrich) in PBS for 40 min at 37°C. The 3-D cultured ECSC were isolated from collagen gels by centrifugation. Whole-cell extracts of 3-D- and 2-D-cultured ECSC were prepared by lysing the cells in lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, and 0.1% phenylmethylsulfonyl fluoride). The suspension was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL). The whole-cell protein extract was resolved with SDS-PAGE using a 10% polyacrylamide gel under reduced conditions. After transfer to Immobilon-P transfer membrane (Millipore, Bedford, MA), the protein was stained with Ponceau S (Sigma-Aldrich) to verify uniform loading and transfer. Membranes were blocked with 5% skim milk (Becton Dickinson) in Tris-buffered saline with Tween 20 (TBS-T) [50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 (pH 7.4)] overnight and subsequently incubated with primary antibodies [α-SMA (1A4; R&D Systems, Minneapolis, MN), Rho A and ROCK-I (Sigma-Aldrich), ROCK-II (Santa...)
Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (E17; Epitomics, Inc., Burlingame, CA), Bcl-X(L (54H6; Cell Signaling, Beverly, MA), p16INK4a (EP4353Y; Epitomics), p21Waf1/Cip1 (12D1; Cell Signaling), and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) (Ambion, Austin, TX) at appropriate dilutions for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analyzed by enhanced chemiluminescence (Amerham Pharmacia Biotech, Chicago, IL). The relative expression of these proteins in ECSC was analyzed using the public domain Image program ImageJ version 1.44.

**Statistical analysis**

Data were calculated as percentages relative to the untreated controls, presented as means ± SD, and appropriately analyzed by the Mann-Whitney U test and the t test with Bonferroni correction using Sigmaplot version 11.2 (Systat Software, Inc., San Jose, CA). Values of P < 0.05 were considered to be statistically significant.

**Results**

**Effects of fasudil on the cell proliferation of ECSC**

The effects of fasudil on the cell proliferation of ECSC were assessed by the WST-1 assay. As shown in Fig. 2, the cell number of viable ECSC was significantly decreased by addition of increasing amounts of fasudil. Cell viability decreased to 88.8 ± 1.0% of the initial value after treatment with fasudil at 0.1 μM (P < 0.0005) and further decreased to 51.7 ± 1.5% after treatment with fasudil at 100 μM (P < 0.0001).

To further assess the effects of fasudil on cell proliferation, DNA synthesis of ECSC after fasudil treatment was evaluated by the BrdU incorporation assay. As shown in Fig. 3, DNA synthesis of ECSC was significantly decreased by the addition of increasing amounts of fasudil. BrdU incorporation decreased to 75.7 ± 0.8% of the initial value after treatment with fasudil at 0.1 μM (P < 0.0001) and further decreased to 56.3 ± 0.6% after treatment with fasudil at 100 μM (P < 0.0001). The inhibitory effects of fasudil on the cell proliferation of ECSC were diminished by the addition of 10% FBS to the experimental media (data not shown).

**Effects of fasudil on the of the cell cycle of ECSC**

The effects of fasudil on the cell cycle of ECSC were determined by flow cytometry. As shown in Fig. 4, culturing of ECSC for 48 h in the presence of fasudil (100 μM) resulted in an accumulation of these cells in the G2/M phase of the cell cycle (4.3 ± 2.1 to 19.2 ± 1.9%, P < 0.0001), with a concomitant decrease in the proportion of these cells in the S phase (45.6 ± 0.8 to 25.0 ± 3.5, P < 0.0001).

**Effects of fasudil on the apoptosis of ECSC**

The apoptotic effects of fasudil on ECSC were assessed by evaluating the presence of internucleosomal DNA fragmentation with a cell death detection ELISA. As shown in Fig. 5, apoptosis of ECSC was significantly induced by the addition of increasing amounts of fasudil. The proportion of apoptotic cells increased to 139.7 ± 4.9% of the baseline value after treatment with fasudil at 1 μM (P < 0.0005) and further increased to 223.3 ± 5.8% after treatment with fasudil at 100 μM (P < 0.0001). The inhibitory effects of fasudil on the apoptosis of ECSC were diminished by
the addition of 10% FBS to the experimental media (data not shown).

Effects of fasudil on ECSC-mediated collagen gel contraction

The effect of fasudil on the contractility of ECSC was evaluated using a collagen gel contraction assay. The surface area of the 35-mm culture dish was defined as 100%. In the presence of 10% FBS, untreated ECSC showed significant collagen gel contractility after 48 h of 3-D culture (relative surface area was $20.8 \pm 0.6\%$ compared with 0-h controls) (Fig. 6A). The contractility of ECSC was significantly attenuated by fasudil in a dose-dependent manner (Relative surface area of ECSC treated with at 100 $\mu$M fasudil was $70.7 \pm 3.4\%$ that of 0-h controls, $P < 0.0001$) (Fig. 6B).

Effects of fasudil on the morphology and the cell density of ECSC in 3-D collagen gel culture

When untreated ECSC were cultured in 3-D collagen gels, the cells adhered to the collagen fibers, and the initially loose network contracted into a dense tissue-like structure (Fig. 7A). The morphology was dendritic to stellate. In contrast, the contractile force of the fasudil-treated ECSC was weak. Moreover, the cell morphology remained round to polygonal, and the cells did not adhere as readily to the collagen fibers as did the untreated ECSC.

In addition to the morphological changes, the cell density of 3-D cultured ECSC was significantly decreased by fasudil treatment at the concentration of 100 $\mu$M (Fig. 7B). The morphology of 3-D cultured ECSC treated with fasudil at 0.1–10 $\mu$M was not examined.

Expression of contraction-related proteins, apoptosis-related proteins, and cell cycle-related proteins in ECSC

To analyze the underlying mechanisms of the action of fasudil on the contractility of ECSC, the expressions of $\alpha$-SMA, RhoA, ROCK-I, and ROCK-II proteins in ECSC were evaluated by fasudil in a dose-dependent manner (Relative surface area of ECSC treated with at 100 $\mu$M fasudil was $70.7 \pm 3.4\%$ that of 0-h controls, $P < 0.0001$) (Fig. 6B).
were evaluated. As shown in Fig. 8, the levels of α-SMA, ROCK-I, and ROCK-II protein in 3-D cultured ECSC were significantly inhibited by the addition of fasudil (100 μM), whereas RhoA expression was not affected by the addition of fasudil.

To analyze the underlying mechanisms of the action of fasudil on the proliferation and apoptosis of ECSC, the expressions of antiapoptotic factors Bcl-2 and Bcl-X<sub>L</sub> and cell cycle modulators p16<sup>INK4a</sup> and p21<sup>Waf1/Cip1</sup> were evaluated. As shown in Fig. 8, the levels of Bcl-2 and Bcl-X<sub>L</sub> expression in 2-D cultured ECSC were strongly inhibited by the addition of fasudil (100 μM), whereas the levels of p16<sup>INK4a</sup> and p21<sup>Waf1/Cip1</sup> expression were strongly up-regulated by the addition of fasudil (100 μM).

**Discussion**

In an effort to clarify the pathological tissue remodeling in endometriosis and to establish novel therapeutic strategies for this enigmatic disease, our laboratory has been conducting an ongoing investigation into the contractile profiles, proliferative activities, and gene expression of endometriotic cells. In the present study, the following points were demonstrated: 1) fasudil inhibits the cell proliferation of ECSC; 2) fasudil induces the cell cycle arrest of ECSC at G2/M phase; 3) fasudil induces the apoptosis of ECSC by down-regulating Bcl-2 and Bcl-X<sub>L</sub> expression; 4) fasudil inhibits the contractility of ECSC in vitro; 5) fasudil inhibits the morphological changes of 3-D cultured ECSC; 6) fasudil decreases the cell density of 3-D cultured ECSC; and 7) fasudil inhibits the differentiation of ECSC into the myofibroblastic phenotype. All of these effects of fasudil were suggested to be mediated by the inhibition of mevalonate-Rho/ROCK-mediated signal pathways and considered to be beneficial for the treatment of endometriosis-associated fibrosis.

Fasudil is now used for a variety of cardiovascular, cerebrovascular, and neurovascular diseases (12–15). This drug is considered to be effective without inducing severe side effects. Fasudil has been reported to inhibit ROCK at an inhibition constant (Ki) value of 0.33 mM (11), more efficiently than myosin light chain kinase (17). It has also been reported that fasudil inhibits ROCK selectively with IC<sub>50</sub> values of 801 and 325 nM for ROCK-I and ROCK-II, respectively (21). It has been reported that the maximum drug concentration (C<sub>max</sub>) for fasudil in 12 healthy elderly volunteers after iv infusion of fasudil (60 mg/60 min) was 1.03 ± 0.21 μM (299.2 ± 62.0 ng/ml) (13). There was no serious adverse event during iv fasudil administration. Clinically, 90 mg/d iv administration of fasudil for 14 d is
considered a therapeutic dose. Our present data demonstrate that fasudil at the concentration of 0.1 μM may affect the proliferation of ECSC, whereas a higher concentration of fasudil was required to induce apoptosis and gel contraction of ECSC. In addition, fasudil is rapidly metabolized in vivo to hydroxyfasudil, which possesses more selective and stronger ROCK-inhibitory effects than its parent drug (15). At 45 min after the initiation of iv infusion, the concentration of hydroxyfasudil present in plasma was approximately 80% that of the parent drug. Hydroxyfasudil is eliminated more slowly than fasudil in vivo. The inhibition activity against ROCK by 0.1 μM fasudil, as used in this in vitro study, would be expected to be close to inhibition by the serum concentration of fasudil plus hydroxyfasudil at a clinical dose.

Members of the Rho family of small guanosine triphosphatases (GTPases) are known to regulate cell growth, morphology, motility, adhesion, migration, and apoptosis, and extracellular matrix contraction through the reorganization of actin cytoskeletons (11, 22–28). The active form of Rho is GTP bound (22, 25), and many polypeptides have been reported as targets of activated Rho, ROCK-I, and ROCK-II. The substrates of the ROCK proteins have been identified, including the myosin phosphatase target-1 subunit, ERN family proteins (ezrin, radixin, and moesin), claudins, adducin, troponin, intermediate filaments (e.g. vimentin and desmin), Na⁺-H⁺ exchangers, and the LIM kinases (Lin11, Isl1, and Mec3) (29–31). It is postulated that these target molecules of fasudil might interact with other signaling pathways, which are known to contribute to the pathogenesis of endometriosis (10).

It has been suggested that the mevalonate-Rho/ROCK pathways play important roles in the formation of endometriosis-associated fibrosis. We demonstrated that simvastatin, which acts as a Rho inhibitor by inhibiting 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase (32), reduced the proliferation and contractility of ECSC (7). Morphological observation of simvastatin-treated ECSC suggested that simvastatin attenuated the attachment of ECSC to collagen fibers, thereby inhibiting the contraction of collagen gels. We have also demonstrated that Y-27632, a pyridine derivative that acts as a specific inhibitor of ROCK-I and ROCK-II (11, 26), significantly inhibited ECSC-mediated contractility (6). Although the precise mechanisms are unknown, it has been demonstrated that heparin (9) and decidualization (8) attenuated the contractility of ECSC. Based on these findings, it can be concluded that agents targeting mevalonate-Rho/ROCK-mediated pathways are promising candidates for the treatment and prevention of endometriosis-associated fibrosis.

Recently, in addition to fasudil and Y-27632, novel ROCK inhibitors with improved potency and selectivity have been developed for clinical use. These molecules include SAR407899 (33), SB-772077B (34), GSK269962A (34), SR-715 (35), SR-899 (35), SLx-2119 (36), and Y-32885 (37). However, fasudil is currently the only clinically available ROCK inhibitor with minimal adverse effects (38). Importantly, fasudil can be administered iv, ip,
and orally (15, 39), which is considered to be beneficial for the long-term treatment of endometriosis. Although additional animal and clinical studies are necessary, our present findings suggest that targeting Rho-ROCK inhibition by fasudil is a promising therapeutic strategy for treating endometriosis.

Acknowledgments

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