Ciglitizone inhibits cell proliferation in human uterine leiomyoma via activation of store-operated Ca\(^{2+}\) channels

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UTERINE LEIOMYOMAS, also known as myomas or fibroids, are the most common benign tumors in the human female pelvis and the leading indicator for hysterectomies (9). Common symptoms associated with these tumors are pain, discomfort, menstrual disturbances, and infertility (7). The cause of uterine leiomyomas is unknown, but the growth of these tumors is thought to be modulated by the ovarian hormones estrogen and progesterone (21, 30). Several apoptosis inducers were shown to activate a Ca\(^{2+}\)-permeable cation channel (15), and Ca\(^{2+}\) influx blockers were found to suppress the inhibition of cell proliferation and metastatic potential of cancer cell lines (32). Therefore, apoptosis has become a molecular targeted therapy for cancer.

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Additionally, abnormal Ca\(^{2+}\) signaling is a central feature of tumor cells and therefore a potential targeted therapy for cancer (17, 19, 32).

As of this date, no reports have been available on the acute interaction between PPAR-\(\gamma\) and estrogen receptors in terms of cell proliferation and intracellular Ca\(^{2+}\) signaling in normal myometrium and uterine leiomyoma. We believe this study is the first attempt to analyze and demonstrate acute effects of the PPAR-\(\gamma\) ligand ciglitizone on cell proliferation and intracellular Ca\(^{2+}\) signaling in normal myometrium and uterine leiomyoma.

**MATERIALS AND METHODS**

In compliance with the recommendations of the Declaration of Helsinki, each patient was informed of the aims and methods of this study and the anticipated benefits, potential hazards, and discomfort that their participation in the study might entail, as well as of their right to abstain from participating in this study and to withdraw their consent at any time. We obtained each patient’s freely given informed consent in writing before enrolling the patient in the study.

**Materials and solutions.** Dulbecco’s modified Eagle’s medium (DMEM), F-12 nutrient mixture and other supplements for cell culture were obtained from GIBCO-BRL (Grand Island, NY). Ciglitizone (Sigma St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution (100 mM) and then used at the final concentration in the testing solution. Fura-2 acetoxyethyl ester (AM) was obtained from Molecular Probes (Eugene, OR) and dissolved in DMSO. Raloxifene, GW9662 (Alexis, Lausen, Switzerland), cyclopiazonic acid, U-73122, and dantrolene sodium were dissolved in DMSO as a stock solution and then used at the final concentration in the testing solution. All other laboratory chemicals were purchased from Sigma.

Physiological saline solution for measurement of [Ca\(^{2+}\)]\(_i\) contained (in mM) 126 NaCl, 5 KCl, 1 CaCl\(_2\), 1.2 MgCl\(_2\), 10 HEPES, and 10 glucose. The solution was titrated to pH 7.4 with HCl, and the osmolarity was 290 mosM. For Ca\(^{2+}\)-free solution, Ca\(^{2+}\) was removed and 2 mM EGTA was added.

**Cell culture.** After approval from our Institutional Review Board, myometrium and uterine leiomyoma specimens were obtained from patients undergoing elective hysterectomies who gave informed consent. The tissue was minced and washed in an ice-cold solution containing 0.25 M sucrose and 20 mM Tris·HCl (pH 7.2). Isolated myocytes were obtained after inducing two phases of enzymatic digestion at 37°C for 45 min (34). The first digestion solution contained (in mg/ml) 1.5 collagenase-dispase, 1 trypsin inhibitor, and 2 bovine serum albumin in Ca\(^{2+}\)-free Hanks’ solution. The second digestion solution contained (in mg/ml) 1 collagenase-dispase, 0.3 trypsin inhibitor, and 2 bovine serum albumin. The cells were then centrifuged, washed, and resuspended in a culture medium consisting of DMEM supplemented with F-12 nutrient mixture and 10% fetal bovine serum. Cells were plated in six-well plates precoated with poly-L-lysine at a final density of 1.5 \(\times\) 10\(^5\) cells in each well for cell counting. Cells were also plated in 96-well plates precoated with poly-L-lysine at a density of 5 \(\times\) 10\(^3\) cells per well for 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay and plated on poly-L-lysine-coated glass coverslips (25 mm in diameter) at a density of 2 \(\times\) 10\(^3\) cells for measurement of [Ca\(^{2+}\)]\(_i\).

**Cell proliferation assays.** To directly ascertain the number of live cells after ciglitizone treatment, myometrial and leiomyoma cells (1.5 \(\times\) 10\(^5\) cells/well) were plated on a six-well plate in 2 ml of DMEM supplemented with 10% fetal bovine serum and F-12 nutrient mixture. Both types of cells were exposed to ciglitizone for 48 h. At the end of each experiment the cells were trypsinized and pelleted together in the culture medium. After staining for 10 min with 0.2% Trypan blue solution, live ( unstained) and dead (Trypan blue positive) cells were counted in a hemocytometer chamber and presented as the percentage of viable cells out of total counted cells. In addition, cellular viability was evaluated by reducing MTT to formazan. After incubation for 4 h with MTT (0.5 mg/ml) at 37°C, isopropanol-HCl was added to each 96-well plate and the absorbance of solubilized MTT formazan products was measured at 570 nm.

**Measurement of [Ca\(^{2+}\)]\(_i\).** Microfluorescent imaging of [Ca\(^{2+}\)]\(_i\) was performed on myometrium and leiomyoma loaded with the calcium indicator dye fura-2 AM. Some of the procedures used for calcium imaging in this experiment were described previously (2). Fura-2 AM (3 \(\mu\)M) was added to both types of cells bathed in 1.8 mM Ca\(^{2+}\)-containing physiological saline solution at room temperature for 30 min. This was followed by a 30-min wash in dye-free saline solution to allow esterase to convert to the free form of fura-2. Coverslips were placed on the stage of an inverted microscope, and imaging was performed with a dual-wavelength system (Intracellular Imaging, Cincinnati, OH). Change in [Ca\(^{2+}\)]\(_i\) was calculated as the relationship between the ratio of emissions at 510 nm and excitation at 340 and 380 nm, respectively. Ratio images were processed every 5 s and converted to [Ca\(^{2+}\)]\(_i\), compared with a range of such ratios obtained by measurement of fura-2 in the presence of known concentrations of Ca\(^{2+}\) (calcium calibration buffer kit, Molecular Probes). Each experimental data point represents the mean [Ca\(^{2+}\)]\(_i\), calculated from at least 12 individually measured cells from three separate cultures. All imaging experiments were done at room temperature (20–22°C).

**Statistics.** Results are given as means ± SE. Statistical analyses were performed by ANOVA, with significance set at \(P < 0.05\).

**RESULTS**

**Effects of ciglitizone on cell viability and [Ca\(^{2+}\)]\(_i\) in human uterine myometrium and uterine leiomyoma.** The PPAR-\(\gamma\) ligand ciglitizone inhibited the proliferation of uterine leiomyoma to a much greater extent than in myometrium (Fig. 1A). In both types of cells, ciglitizone induced the initial [Ca\(^{2+}\)]\(_i\) increase dose-dependently (Fig. 1B). The initial ciglitizone-induced [Ca\(^{2+}\)]\(_i\) responses were the same in both types of cells regardless of the presence or absence of external Ca\(^{2+}\). However, the secondary [Ca\(^{2+}\)]\(_i\) responses exhibited a discrepancy between myometrium and uterine leiomyoma in the patterns of [Ca\(^{2+}\)]\(_i\) increase. The ciglitizone-induced secondary [Ca\(^{2+}\)]\(_i\) increase was only observed in uterine leiomyoma bathed in external 1.8 mM Ca\(^{2+}\) solution, whereas in the absence of external Ca\(^{2+}\), ciglitizone induced the initial [Ca\(^{2+}\)]\(_i\) increase without a second increase (Fig. 1, C and D).

**Ciglitizone-induced initial intracellular Ca\(^{2+}\) mobilization in human uterine myometrium and leiomyoma.** Experiments were performed to explore whether sarcoplasmic reticulum Ca\(^{2+}\) stores are involved in ciglitizone-induced Ca\(^{2+}\) release. In the absence of external Ca\(^{2+}\), ciglitizone demonstrated a typical [Ca\(^{2+}\)]\(_i\) increase as in previous experiments with cells bathed in 1.8 mM external Ca\(^{2+}\) solution. Ciglitizone-induced initial peak [Ca\(^{2+}\)]\(_i\) values were not different in myometrium (97.5 ± 6.34 nM, \(n = 28\)) and leiomyoma (107.2 ± 6.93 nM, \(n = 25\)). However, after the initial [Ca\(^{2+}\)]\(_i\) increase, cotreatment with 10 \(\mu\)M cyclopiazonic acid [an inhibitor of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase] did not increase [Ca\(^{2+}\)]\(_i\) (Fig. 2A). Conversely, the first treatment of cyclopiazonic acid actually led to depletion of the stored Ca\(^{2+}\) in the sarcoplasmic reticulum and caused a transient [Ca\(^{2+}\)]\(_i\) increase. Cyclopiazonic acid-induced initial peak [Ca\(^{2+}\)]\(_i\) values were not different in myometrium (82.2 ± 4.26 nM, \(n = 34\)) and leiomyoma (92.1 ± 6.26 nM, \(n = 30\)). After this response, 100 \(\mu\)M ciglitizone was added, but it did not induce another increase of [Ca\(^{2+}\)]\(_i\) in either type of cell (Fig. 2B). In Ca\(^{2+}\)-free solution, pretreatment with 5 \(\mu\)M U-73122 [a phospholipase C

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inhibitor and subsequent activator of inositol 1,4,5-trisphosphate (IP₃)-induced Ca²⁺ release did not alter the typical [Ca²⁺]ᵢ increase induced by subsequently adding 100 μM ciglitizone in either uterine leiomyoma and myometrium (Fig. 2C). Under U-73122 pretreatment, ciglitizone-induced initial peak [Ca²⁺]ᵢ values were not different in myometrium (154.2 ± 4.80 nM, n = 27) and leiomyoma (172.8 ± 8.39 nM, n = 27). The same approach was used to study the role of ryanodine receptors in ciglitizone-induced [Ca²⁺]ᵢ increase. After pretreatment with 15 μM dantrolene sodium, a ryanodine receptor blocker, the ciglitizone-induced [Ca²⁺]ᵢ increases were completely blocked in both types of cells (Fig. 2D) and peak [Ca²⁺]ᵢ values were not different in myometrium (49.7 ± 3.51 nM, n = 24) and leiomyoma (53.8 ± 4.10 nM, n = 21).
Effects of antagonists for PPAR-γ, estrogen receptors, and store-operated Ca^{2+} channels on ciglitzone-induced secondary [Ca^{2+}i] increases and cell proliferation in primary cultured human uterine leiomyoma. Ciglitzone-induced initial peak [Ca^{2+}i] values were not different under control conditions (144.3 ± 5.12 nM, n = 23) and under GW9662 pretreatment (150.7 ± 4.18 nM, n = 32) in uterine leiomyoma cells. After pretreatment with 30 μM GW9662 (PPAR-γ antagonist), the ciglitzone-induced [Ca^{2+}i], secondary increasing plateau values, measured during the last 30 s of each experiment, were significantly decreased to 127.4 ± 5.68 nM compared with 178.9 ± 8.16 nM in control cells (P < 0.001), whereas the selective estrogen receptor modulator raloxifene (20 μM) decreased the ciglitzone-induced initial [Ca^{2+}i] increase [188.1 ± 5.38 nM (n = 28)] vs. 122.9 ± 4.15 nM (n = 29); P < 0.001 and the secondary plateau value (316.7 ± 10.20 nM vs. 91.9 ± 4.62 nM; P < 0.001) in uterine leiomyoma (Fig. 3, A and B). To establish the source of the secondary [Ca^{2+}i], enhancement in uterine leiomyoma, two store-operated Ca^{2+} channel (SOCC) blockers, SKF-96365 and lanthanum chloride, were used. The ciglitzone-induced secondary [Ca^{2+}i] increase in uterine leiomyoma was not prevented by 10 μM SKF-96365 and 3,5-bistrifluoromethyl pyrazole (BTP2), a specific Ca^{2+} release-activated Ca^{2+} channel blocker (data not shown), whereas lanthanum chloride blocked the Ca^{2+}i increase in a concentration-dependent manner (Fig. 3C). Ciglitzone-induced initial peak [Ca^{2+}i] values were not different under control conditions (190.0 ± 10.92 nM, n = 32) and under 50 μM lanthanum treatment (222.4 ± 14.10 nM, n = 31), but 100 μM lanthanum decreased the initial peak values to 155.9 ± 8.72 nM (n = 31; P < 0.001). In the presence of lanthanum, the secondary plateau values were significantly decreased under 50 μM (141.3 ± 4.08 nM; P < 0.001) and 100 μM (72.1 ± 2.36 nM; P < 0.001) lanthanum compared with control (239.4 ± 6.82 nM). Raloxifene (P < 0.001), lanthanum chloride (P < 0.001), and GW9662 (P < 0.01), which blocked the ciglitzone-induced secondary [Ca^{2+}i], increase, significantly restored the cell proliferation inhibited by ciglitzone treatment in uterine leiomyoma just as they had in the calcium experiments (Fig. 3D).

Effects of exposure time of ciglitzone and lanthanum on cell proliferation in human uterine leiomyoma. To correlate the ciglitzone-induced short-term changes in [Ca^{2+}i], with the long-term changes in cell proliferation, we observed the time dependence of ciglitzone and the effects of lanthanum on cell viability in human uterine leiomyoma (Fig. 4). Ciglitzone (100 μM) decreased cell viability in a time-dependent manner. In accordance with an attenuating effect of lanthanum on the ciglitzone-induced secondary [Ca^{2+}i], increase as shown in Fig. 3C, these decrements of ciglitzone-induced cell viability were significantly (P < 0.001) recovered after 4 h of cotreatment with lanthanum and ciglitzone compared with values in the corresponding time for treatment with ciglitzone alone.

Effects of extracellular and intracellular free Ca^{2+} concentrations on cell proliferation in human uterine leiomyoma. To establish the relationship between an activation of SOCCs and a decrement of cell proliferation, the extracellular free Ca^{2+} concentration was decreased by adding EGTA and the effects of EGTA on cell viability were evaluated in culture medium (Fig. 5A). In the presence of EGTA, ciglitzone-induced cell proliferation was increased in a dose-dependent manner up to 1 mM EGTA and significantly increased compared with the viability in treatment with ciglitzone alone (P < 0.001). Ciglitzone induced Ca^{2+} release via an activation of the ryanodine receptor of the sarcoplasmic reticulum (Fig. 2D) and Ca^{2+} influx via an activation of SOCCs of the plasma membrane (Fig. 3C). Therefore, we also evaluated the effects of ryanodine receptor agonist and Ca^{2+} influx on cell prolifera-
Ciglitizone induces initial [Ca\textsuperscript{2+}] increase in myometrium and leiomyoma. These initial [Ca\textsuperscript{2+}] increases resulted from the release of Ca\textsuperscript{2+} from the ryanodine receptor of the sarcoplasmic reticulum. This conclusion was based on the following three observations. First, the initial [Ca\textsuperscript{2+}] increase was triggered by ciglitizone even in external Ca\textsuperscript{2+} free solution containing EGTA. Second, prior depletion of intracellular Ca\textsuperscript{2+} stores by the sarco(endo)plasmic Ca\textsuperscript{2+}-ATPase inhibitor cyclopiazonic acid prevented the ciglitizone-induced [Ca\textsuperscript{2+}] increase. Third, dantrolene sodium, a ryanodine receptor inhibitor, blocked the ciglitizone-induced initial [Ca\textsuperscript{2+}] increase in myometrium and leiomyoma.

The two primary pathways for obtaining stimulus-increased levels of [Ca\textsuperscript{2+}] in smooth muscle cells are release from sarcoplasmic reticulum stores and an influx from extracellular space (23). The increased [Ca\textsuperscript{2+}] levels observed in myometrium and uterine leiomyoma could be from the sarcoplasmic reticulum. Release of Ca\textsuperscript{2+} from intracellular stores into the cytoplasm occurs through two classes of Ca\textsuperscript{2+} release channels, IP\textsubscript{3} receptors and ryanodine receptors, which may or may not coexist, depending on the tissue (5). Several mechanisms of intracellular Ca\textsuperscript{2+} mobilization are present in uterine myometrium. However, in human uterine myometrium only IP\textsubscript{3}-induced Ca\textsuperscript{2+} release has been extensively studied to date (27). The presence of functional ryanodine receptors was also established, and cADP-ribose served as an endogenous regulator for human myometrial Ca\textsuperscript{2+} regulation (10, 34). Although IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release by a contractant such as oxytocin is a popular mechanism for increasing intracellular Ca\textsuperscript{2+} in myometrium (27), this type of Ca\textsuperscript{2+} release was not involved in this ciglitizone-induced [Ca\textsuperscript{2+}] experiment. This study observed that the ryanodine receptor blocker dantrolene sodium completely suppressed the ciglitizone-induced initial [Ca\textsuperscript{2+}] increase, whereas the IP\textsubscript{3}-receptor blocker U-73122 did not have an effect on the [Ca\textsuperscript{2+}], response. These results suggested that the pathways of ciglitizone-induced initial [Ca\textsuperscript{2+}] increase are the ryanodine receptors of the sarcoplasmic reticulum in human uterine myometrium and leiomyoma.

In this study, the initial [Ca\textsuperscript{2+}] increase induced by ciglitizone was inhibited by raloxifene, but the secondary [Ca\textsuperscript{2+}] increase was attenuated by the PPAR-\gamma antagonists. These findings suggest that ciglitizone induced a rapid initial [Ca\textsuperscript{2+}] increase via estrogen receptors and that this response was inhibited by raloxifene. Recently, Houston et al. (16) were the first to demonstrate that ciglitizone inhibits cell proliferation stimulated by estradiol and that stimulation of PPAR-\gamma signaling also inhibits estrogen receptor-mediated gene expression of vit-ERE-Luc reporter and progesterone receptor A. The results show that ciglitizone inhibited the initial [Ca\textsuperscript{2+}] release from the sarcoplasmic reticulum through estrogen receptors in uterine leiomyoma, as shown in Fig. 3B.

**Discussion**

Effects of ciglitizone on [Ca\textsuperscript{2+}] increase and intracellular Ca\textsuperscript{2+} mobilization in human uterine myometrium and leiomyoma cells. This study first indicated that the PPAR-\gamma ligand ciglitizone induces [Ca\textsuperscript{2+}] increases via an initial activation of intracellular Ca\textsuperscript{2+} release in myometrium and uterine leiomyoma and a secondary activation of SOCCs in leiomyoma. These initial [Ca\textsuperscript{2+}] increases resulted from the release of Ca\textsuperscript{2+} from the ryanodine receptor of the sarcoplasmic reticulum. This conclusion was based on the following three observations. First, the initial [Ca\textsuperscript{2+}] increase was triggered by ciglitizone even in external Ca\textsuperscript{2+} free solution containing EGTA. Second, prior depletion of intracellular Ca\textsuperscript{2+} stores by the sarco(endo)plasmic Ca\textsuperscript{2+}-ATPase inhibitor cyclopiazonic acid prevented the ciglitizone-induced [Ca\textsuperscript{2+}] increase. Third, dantrolene sodium, a ryanodine receptor inhibitor, blocked the ciglitizone-induced initial [Ca\textsuperscript{2+}] increase in myometrium and leiomyoma.

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In this study, the initial [Ca\textsuperscript{2+}] increase induced by ciglitizone was inhibited by raloxifene, but the secondary [Ca\textsuperscript{2+}] increase was attenuated by the PPAR-\gamma antagonists. These findings suggest that ciglitizone induced a rapid initial [Ca\textsuperscript{2+}] increase via estrogen receptors and that this response was inhibited by raloxifene. Recently, Houston et al. (16) were the first to demonstrate that ciglitizone inhibits cell proliferation stimulated by estradiol and that stimulation of PPAR-\gamma signaling also inhibits estrogen receptor-mediated gene expression of vit-ERE-Luc reporter and progesterone receptor A. The results show that ciglitizone inhibited the initial [Ca\textsuperscript{2+}] release from the sarcoplasmic reticulum through estrogen receptors in uterine leiomyoma, as shown in Fig. 3B.

Ciglitizone-induced SOCC activation in human uterine leiomyoma. Despite the same initial [Ca\textsuperscript{2+}], increase in both types of cells, the ciglitizone-induced secondary [Ca\textsuperscript{2+}] in-
crease was only observed in uterine leiomyoma cells bathed with 1.8 mM Ca\(^{2+}\)-containing solution and was blocked in the presence of lanthanum. Therefore, ciglitzone triggered the secondary Ca\(^{2+}\) rise with an influx of extracellular Ca\(^{2+}\), as well as an initial [Ca\(^{2+}\)]\(_i\) increase from intracellular Ca\(^{2+}\) stores. The influx of Ca\(^{2+}\) from extracellular space occurs through three types of Ca\(^{2+}\) gates: voltage-dependent Ca\(^{2+}\) channels, ligand-gated Ca\(^{2+}\) channels, and SOCCs, also termed capacitative Ca\(^{2+}\) entry channels (27).

Activation of Ca\(^{2+}\) release pathways from the sarcoplasmic reticulum leads to the depletion of intracellular Ca\(^{2+}\) stores after sustained activation of IP\(_3\) or ryanodine receptors. This signal induces Ca\(^{2+}\) influx via activation of SOCCs to mediate the replenishing of sarcoplasmic reticulum Ca\(^{2+}\) (13, 20, 26). SOCC entry can be induced as a result of the specific inhibition of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase by agents such as cyclopiazonic acid and thapsigargin (12). Several findings provide evidence for SOCC entry in human myometrium cells (27). This SOCC entry can be blocked by the removal of extracellular Ca\(^{2+}\) or channel antagonists including lanthanum and SKF-96365 (13, 35). We also tested the effects of BTP2, a specific blocker of the Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel that is one type of SOCC in T lymphocytes (36), on ciglitzone-induced [Ca\(^{2+}\)]\(_i\) increase and cell proliferation in uterine leiomyoma, but BTP2 did not affect the [Ca\(^{2+}\)]\(_i\); increase and cell viability like SKF-96365 (data not shown). The actions of SOCC inhibitors are dependent on the tissue, which may be explained by the existence of multiple types of SOCCs (13). For example, the activation of SOCC entry after intracellular Ca\(^{2+}\) depletion was prevented by SKF-96365 and lanthanum in central nervous system neurons (1). In cerebral arteriolar smooth muscle cells, SOCC influx was completely resistant to SKF-96365 but was inhibited by lanthanum (13), which was in accordance with the behavior of uterine leiomyoma in the present study.

Ciglitzone-induced cellular apoptosis in human uterine leiomyoma. Ca\(^{2+}\) is an important secondary messenger that plays an integral role in a variety of cellular processes, including suppression of Bcl-2 (an antiapoptotic protein), which would initiate a program of events leading to cell death (3, 20). We also observed that a high dose of ciglitzone caused apoptosis in primary cultured human uterine leiomyoma (unpublished observation). In addition to apoptosis, very high levels of Ca\(^{2+}\) can lead to necrosis through the activity of Ca\(^{2+}\)-sensitive protein-digesting enzymes (3). In this study, we found that lanthanum chloride, raloxifene, and GW9662 inhibited the ciglitzone-induced secondary [Ca\(^{2+}\)]\(_i\) increase via activation of SOCC and recovered the inhibition of cell proliferation compared with treatment with ciglitzone alone. Therefore, ciglitzone-induced [Ca\(^{2+}\)]\(_i\) and cell proliferation data provide evidence for an interaction between the activation of SOCC and the cell proliferation in uterine leiomyoma. Because abnormal Ca\(^{2+}\) is also a central feature of tumor cells (32), the PPAR-\(\gamma\) ligand ciglitzone could be examined as part of a new therapeutic strategy in dealing with human leiomyoma.

In conclusion, the results of this study suggest that ciglitzone inhibits cell proliferation and increases [Ca\(^{2+}\)]\(_i\) through the activation of SOCCs, especially in human uterine leiomyoma.

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