Cigliotizone 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. This hormone-dependent leiomyoma growth is demonstrated by the fact that most of these tumors are diagnosed during reproductive periods, changes in size during pregnancy, or regress after the onset of menopause, which coincide with dramatic changes in hormonal secretion (7).

Estrogen is believed to be one of the most important hormones affecting uterine contractility during pregnancy. It also plays a critical role in both uterine and vaginal growth, as well as in adult function. Estrogen elicits its effect via estrogen receptors found in estrogen-responsive tissues (11). Estrogen triggers genomic effects via interaction with specific intracellular nuclear receptors and also produces acute effects by acting on plasma membrane receptors through nongenomic processes (11, 25). Recently, Houston et al. (16) demonstrated that in uterine leiomyomas peroxisome proliferator-activated receptor (PPAR)-γ activation induced inhibition of cell proliferation via genomic effects and showed that this inhibition was mediated at least in part by negative cross-talk between estrogen receptors and PPAR signaling pathways.

PPARs are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, which have a role in many cellular functions, including lipid metabolism, cell proliferation, differentiation, adipogenesis, and inflammatory signaling (18, 33). PPAR-γ, one of three mammalian PPAR isoforms (α, β/δ, and γ), is found in adipose tissues and other insulin-responsive tissues such as skeletal muscle and liver (24, 29). PPAR-γ mediates adipocyte differentiation and glucose and lipid metabolism (33). Activation of PPAR-γ induces the differentiation and inhibits the proliferation of many types of cancer cells (14, 28). Although there are some reports demonstrating that the three PPAR isoforms are expressed in normal myometrium and uterine leiomyoma at the same level (16) or have a higher incidence in leiomyoma than uterine myometrium (31), the PPAR signal pathway has not been clearly characterized in uterine leiomyoma.

Intracellular Ca\(^{2+}\) regulates a variety of cellular processes (4). An increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{i}\)]) is a pivotal signal for the regulation of cellular function and growth (6). Changes in [Ca\(^{2+}\)\(_{i}\)] stimulate a number of intracellular events and also trigger a cell death process. Evidence shows that a prolonged increase of [Ca\(^{2+}\)\(_{i}\)] leads to apoptosis (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 ciglitzone 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). Ciglitzone (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 osmolality 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. In writing before enrolling the patient in the study.

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

**RESULTS**

Effects of ciglitzone on cell viability and [Ca\(^{2+}\)]\(i\), in human uterine myometrium and uterine leiomyoma. The PPAR-\(\gamma\) ligand ciglitzone inhibited the proliferation of uterine leiomyoma to a much greater extent than in myometrium (Fig. 1A). In both types of cells, ciglitzone induced the initial [Ca\(^{2+}\)]\(i\), increase dose-dependently (Fig. 1B). The initial ciglitzone-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 ciglitzone-induced secondary [Ca\(^{2+}\)]\(i\), increase was only observed in uterine leiomyoma bathed in external 1.8 mM Ca\(^{2+}\), whereas in the absence of external Ca\(^{2+}\), ciglitzone induced the initial [Ca\(^{2+}\)]\(i\), increase without a second increase (Fig. 1, C and D).

Ciglitzone-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 ciglitzone-induced Ca\(^{2+}\), release. In the absence of external Ca\(^{2+}\), ciglitzone demonstrated a typical [Ca\(^{2+}\)]\(i\), increase as in previous experiments with cells bathed in 1.8 mM external Ca\(^{2+}\), solution. Ciglitzone-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 sarc(endo)plasmic 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 ciglitzone 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...
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 Ca2+ channels on ciglitizone-induced secondary [Ca2+]i increases and cell proliferation in human uterine leiomyoma. Ciglitizone-induced initial peak [Ca2+]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 ciglitizone-induced secondary [Ca2+]i response was significantly decreased (P < 0.001), whereas the selective estrogen receptor modulator raloxifene (20 μM) decreased the ciglitizone-induced initial [Ca2+]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 [Ca2+]i enhancement in uterine leiomyoma, two store-operated Ca2+ channel (SOCC) blockers, SKF-96365 and lanthanum chloride, were used. The ciglitizone-induced secondary [Ca2+]i increase in uterine leiomyoma was not prevented by 10 μM SKF-96365 and 3,5-bistrifluoromethyl pyrazole (BTP2), a specific Ca2+ release-activated Ca2+ channel blocker (data not shown), whereas lanthanum chloride blocked the Ca2+ increase in a concentration-dependent manner (Fig. 3C). Ciglitizone-induced initial peak [Ca2+]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 ciglitizone-induced secondary [Ca2+]i increase, significantly restored the cell proliferation inhibited by ciglitizone treatment in uterine leiomyoma just as they had in the calcium experiments (Fig. 3D).

Effects of exposure time of ciglitizone and lanthanum on cell proliferation in human uterine leiomyoma. To correlate the ciglitizone-induced short-term changes in [Ca2+]i with the long-term changes in cell proliferation, we observed the time dependence of ciglitizone and the effects of lanthanum on cell viability in human uterine leiomyoma (Fig. 4). Ciglitizone (100 μM) decreased cell viability in a time-dependent manner. In accordance with an attenuating effect of lanthanum on the ciglitizone-induced secondary [Ca2+]i increase as shown in Fig. 3C, these decrements of ciglitizone-induced cell viability were significantly (P < 0.001) recovered after 4 h of cotreatment with lanthanum and ciglitizone compared with values in the corresponding time for treatment with ciglitizone alone.

Effects of extracellular and intracellular free Ca2+ 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 Ca2+ 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, ciglitizone-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 ciglitizone alone (P < 0.001). Ciglitizone induced Ca2+ release via an activation of the ryanodine receptor of the sarcoplasmic reticulum (Fig. 2D) and Ca2+ influx via an activation of SOCC of the plasma membrane (Fig. 3C). Therefore, we also evaluated the effects of ryanodine receptor agonist and Ca2+ influx on cell prolifera-
Ciglitizone induces \([\text{Ca}^{2+}]\) increases in uterine leiomyoma. Cell viability was analyzed with MTT reduction assay at the time periods indicated. Control cells were treated with vehicle only. Data are mean ± SE values from 3 separate experiments. *P < 0.01, **P < 0.001 vs. treatment with ciglitizone alone.

DISCUSSION

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

The two primary pathways for obtaining stimulus-increased levels of \([\text{Ca}^{2+}]\) in smooth muscle cells are release from sarcoplasmic reticulum stores and an influx from extracellular space (23). The increased \([\text{Ca}^{2+}]\) levels observed in myometrium and uterine leiomyoma could be from the sarcoplasmic reticulum. Release of \([\text{Ca}^{2+}]\) from intracellular stores into the cytoplasm occurs through two classes of \([\text{Ca}^{2+}]\) release channels, IP3 receptors and ryanodine receptors, which may or may not coexist, depending on the tissue (5). Several mechanisms of intracellular \([\text{Ca}^{2+}]\) mobilization are present in uterine myometrium. However, in human uterine myometrium only IP3-induced \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) regulation (10, 34). Although IP3-mediated \([\text{Ca}^{2+}]\) release by a contractant such as oxytocin is a popular mechanism for increasing intracellular \([\text{Ca}^{2+}]\) in myometrium (27), this type of \([\text{Ca}^{2+}]\) release was not involved in this ciglitizone-induced \([\text{Ca}^{2+}]\) experiment. This study observed that the ryanodine receptor blocker dantrolene sodium completely suppressed the ciglitizone-induced initial \([\text{Ca}^{2+}]\) increase, whereas the IP3-receptor blocker U-73122 did not have an effect on the \([\text{Ca}^{2+}]\) response. These results suggested that the pathways of ciglitizone-induced initial \([\text{Ca}^{2+}]\) increase are the ryanodine receptors of the sarcoplasmic reticulum in human uterine myometrium and leiomyoma. In this study, the initial \([\text{Ca}^{2+}]\) increase induced by ciglitizone was inhibited by raloxifene, but the secondary \([\text{Ca}^{2+}]\) increase was attenuated by the PPAR-\(\gamma\) antagonists. These findings suggest that ciglitizone induced a rapid initial \([\text{Ca}^{2+}]\) increase, 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 are supportive of a functional interrelationship between PPAR-\(\gamma\) and the estrogen receptor. The present study also showed that ciglitizone induced the initial \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) increase, increase in both types of cells, the ciglitizone-induced secondary \([\text{Ca}^{2+}]\) in-
crease was only observed in uterine leiomyoma cells bathed with 1.8 mM Ca²⁺-containing solution and was blocked in the presence of lanthanum. Therefore, ciglitizone triggered the secondary Ca²⁺ rise with an influx of extracellular Ca²⁺ as well as an initial [Ca²⁺]i increase from intracellular Ca²⁺ stores. The influx of Ca²⁺ from extracellular space occurs through three types of Ca²⁺ gates: voltage-dependent Ca²⁺ channels, ligand-gated Ca²⁺ channels, and SOCCs, also termed capacitative Ca²⁺ entry channels (27).

Activation of Ca²⁺ release pathways from the sarcoplasmic reticulum leads to the depletion of intracellular Ca²⁺ stores after sustained activation of IP₃ or ryanodine receptors. This signal induces Ca²⁺ influx via activation of SOCCs to mediate the replenishing of sarcoplasmic reticulum Ca²⁺ (13, 20, 26). SOCC entry can be induced as a result of the specific inhibition of sarcoplasmic reticulum Ca²⁺-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 Ca²⁺ or channel antagonists including lanthanum and SKF-96365 (13, 35). We also tested the effects of BTP2, a specific blocker of the Ca²⁺ release-activated Ca²⁺ channel that is one type of SOCC in T lymphocytes (36), on ciglitizone-induced [Ca²⁺]i increase and cell proliferation in uterine leiomyoma, but BTP2 did not affect the [Ca²⁺]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 Ca²⁺ 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.

Ciglitizone-induced cellular apoptosis in human uterine leiomyoma. Ca²⁺ is an important secondary messenger that plays an integral role in a variety of cellular processes, including signal transduction, gene expression, cell proliferation and apoptosis, and muscle excitation-contraction coupling (8, 20). At the cellular level, Ca²⁺ can be either a life signal or a death signal, because changes in cytosolic free Ca²⁺ level and Ca²⁺ content of intracellular stores can control cell growth and proliferation or induce programmed cell death (20).

Sustained increases of intracellular Ca²⁺ to the micromolar range are obviously deleterious to the signaling function of Ca²⁺ (8). In this study, the sustained increase in uterine leiomyoma of [Ca²⁺]i could be expected for a long time via an activation of SOCC entry. Growing evidence suggests that Ca²⁺ plays a pivotal role in apoptosis, which is related to an activation of SOCCs (20, 22). The panoramic picture of Ca²⁺ signaling in apoptosis consists of Ca²⁺ release from sarcoplasmic or endoplasmic reticulum, depletion of intracellular Ca²⁺ stores, and subsequent activation of SOCC entry. Under these conditions, there would be two main consequences for the cell. First, the depletion of Ca²⁺ in sarcoplasmic or endoplasmic reticulum would lead to an activation of stress signals, which switch on the cell death-associated genes. Second, if the mitochondria became overloaded with Ca²⁺, the impact would be reflected by an abnormal mitochondrial metabolism 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 ciglitizone caused apoptosis in primary cultured human uterine leiomyoma (unpublished observation). In addition to apoptosis, very high levels of Ca²⁺ can lead to necrosis through the activity of Ca²⁺-sensitive protein-digesting enzymes (3). In this study, we found that lanthanum chloride, raloxifene, and GW9662 inhibited the ciglitizone-induced secondary [Ca²⁺]i increase via activation of SOCC and recovered the inhibition of cell proliferation compared with treatment with ciglitizone alone. Therefore, ciglitizone-induced [Ca²⁺]i and cell proliferation data provide evidence for an interaction between the activation of SOCC and the cell proliferation in uterine leiomyoma. Because abnormal Ca²⁺ is also a central feature of tumor cells (32), the PPAR-γ ligand ciglitizone could be examined as part of a new therapeutic strategy in dealing with human leiomyoma.

In conclusion, the results of this study suggest that ciglitizone inhibits cell proliferation and increases [Ca²⁺]i, through the activation of SOCCs, especially in human uterine leiomyoma.

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