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

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peroxisome proliferator-activated receptor-γ; intracellular calcium; uterine cells

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-γ 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-γ 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 acetoxymethyl 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-D-lysine at a final density of 1.5×10^6 cells in each well for cell counting. Cells were also plated in 96-well plates precoated with poly-D-lysine at a density of 5×10^4 cells per well for 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay and plated on poly-D-lysine-coated glass coverslips (25 mm in diameter) at a density of 2×10^5 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 (1.5×10^6 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 μM) 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-γ ligand ciglitizone inhibited the proliferation of uterine leiomyoma to a much greater extent than in myometrium (Fig. 1*A*). In both types of cells, ciglitizone induced the initial $[Ca^{2+}]_i$ increase dose-dependently (Fig. 1*B*). 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 μM cyclopiazonic acid [an inhibitor of sarco-(endo)plasmic reticulum Ca^{2+}-ATPase] did not increase $[Ca^{2+}]_i$ (Fig. 2*A*). 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 μM ciglitizone was added, but it did not induce another increase of $[Ca^{2+}]_i$ in either type of cell (Fig. 2*B*). In Ca^{2+}-free solution, pretreatment with 5 μM U-73122 [a phospholipase C

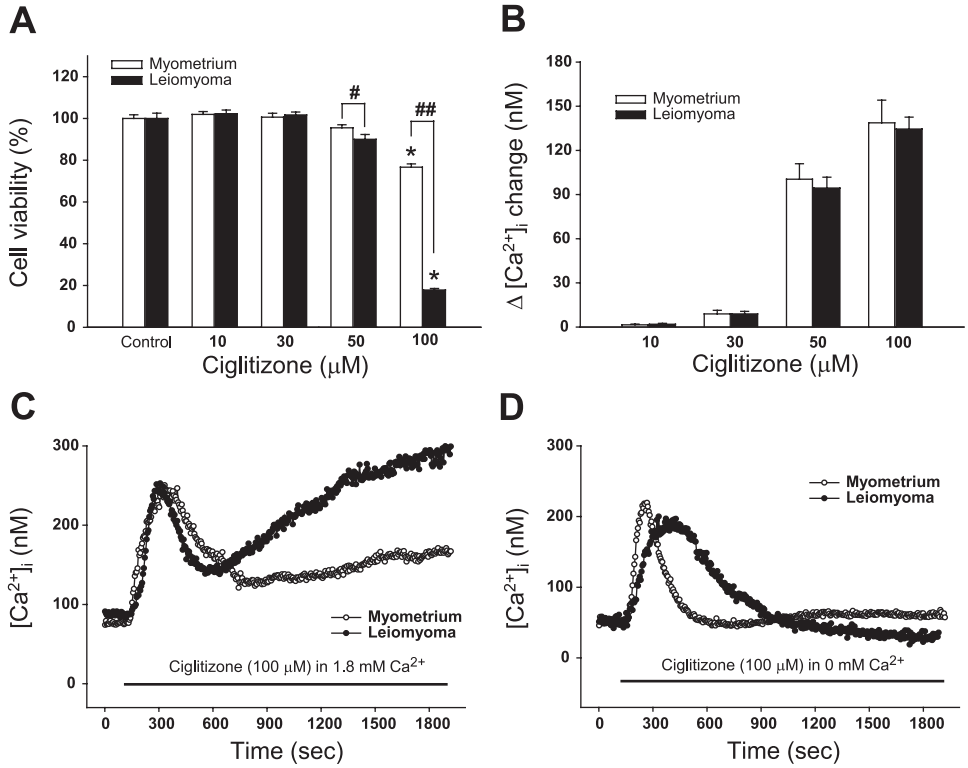

Fig. 1. Effects of the peroxisome proliferator-activated receptor (PPAR)-γ ligand ciglitizone on cell viability and intracellular calcium concentration ($[Ca^{2+}]_i$) in primary cultured human uterine myometrium and uterine leiomyoma. *A*: cell viability was determined by counting cell numbers 48 h after ciglitizone treatment. Control cells were only treated with vehicle. Data are presented as mean ± SE values from 3 separate experiments. *$P < 0.001$ vs. control; #$P < 0.01$, ##$P < 0.001$ vs. myometrium. *B*: dose response of ciglitizone on $[Ca^{2+}]_i$ increases was measured at initial peak values in both types of cells bathed in 1.8 mM Ca^{2+} solution. Effects of external Ca^{2+} on ciglitizone-induced $[Ca^{2+}]_i$ increases in the presence (*C*) or absence (*D*) of 1.8 mM Ca^{2+} are also shown. These data represent the means of values measured in 30–40 cells in 3 separate experiments.

inhibitor and subsequent activator of inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release] did not alter the typical $[Ca^{2+}]_i$ increase induced by subsequently adding 100 μM ciglitizone in either uterine leiomyoma and myometrium (Fig. 2*C*). Under U-73122 pretreatment, ciglitizone-induced initial peak $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increase. After pretreatment with 15 μM dantrolene sodium, a ryanodine receptor blocker, the ciglitizone-induced $[Ca^{2+}]_i$ increases were completely blocked in both types of cells (Fig. 2*D*) and peak $[Ca^{2+}]_i$ values were not different in myometrium (49.7 ± 3.51 nM, $n = 24$) and leiomyoma (53.8 ± 4.10 nM, $n = 21$).

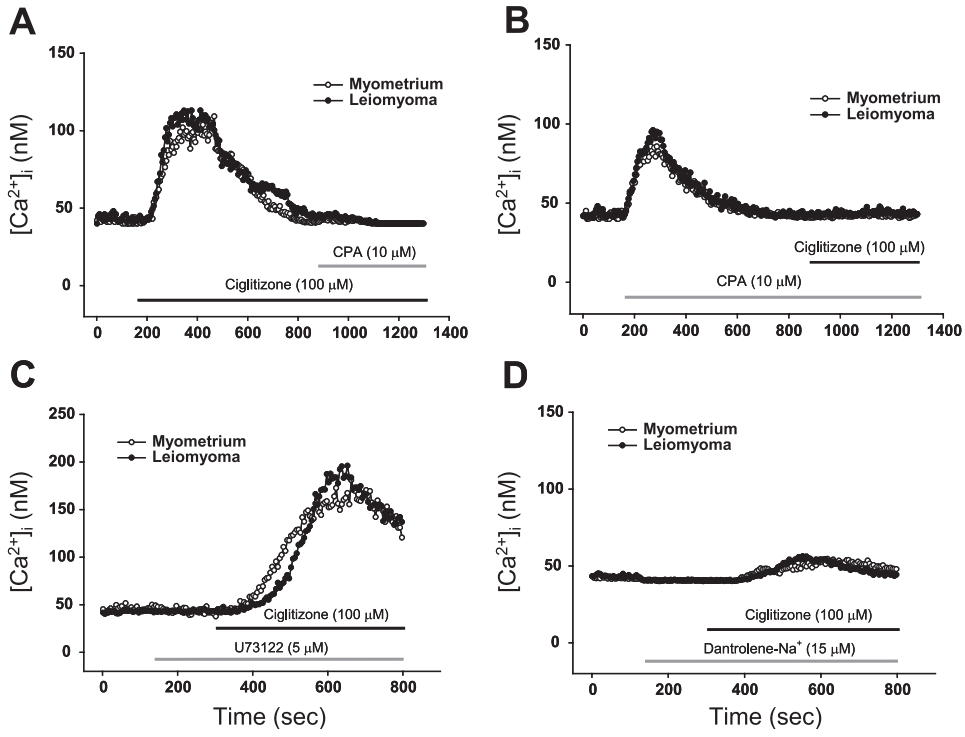

Fig. 2. Intracellular mobilization of ciglitizone-induced initial $[Ca^{2+}]_i$ increases in primary cultured human uterine myometrium and leiomyoma. All experiments were performed in the absence of external Ca^{2+} with 2 mM EGTA. To determine the effects of an inhibitor of sarco(endo)plasmic reticulum Ca^{2+}-ATPase, cyclopiazonic acid (CPA), on ciglitizone-induced $[Ca^{2+}]_i$ increases, CPA was added after (*A*) and before (*B*) ciglitizone treatment. The phospholipase C inhibitor U-73122 (*C*) or the ryanodine receptor blocker dantrolene sodium (*D*) was added to determine their effects on ciglitizone-induced initial Ca^{2+} release from the sarcoplasmic reticulum. These data represent the means of values measured in 30–40 cells in 3 separate experiments.

Effects of antagonists for PPAR-γ, estrogen receptors, and store-operated Ca^{2+} channels on ciglitizone-induced secondary $[Ca^{2+}]_i$ increases and cell proliferation in human uterine leiomyoma. Ciglitizone-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 ciglitizone-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 ciglitizone-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 ciglitizone-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+} increase in a concentration-dependent manner (Fig. 3*C*). Ciglitizone-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 ciglitizone-induced secondary $[Ca^{2+}]_i$ increase, significantly restored the cell proliferation inhibited by ciglitizone treatment in uterine leiomyoma just as they had in the calcium experiments (Fig. 3*D*).

Effects of exposure time of ciglitizone and lanthanum on cell proliferation in human uterine leiomyoma. To correlate the ciglitizone-induced short-term changes in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increase as shown in Fig. 3*C*, 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 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. 5*A*). 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 Ca^{2+} release via an activation of the ryanodine receptor of the sarcoplasmic reticulum (Fig. 2*D*) and Ca^{2+} influx via an activation of SOCC of the plasma membrane (Fig. 3*C*). Therefore, we also evaluated the effects of ryanodine receptor agonist and Ca^{2+} influx on cell prolifera-

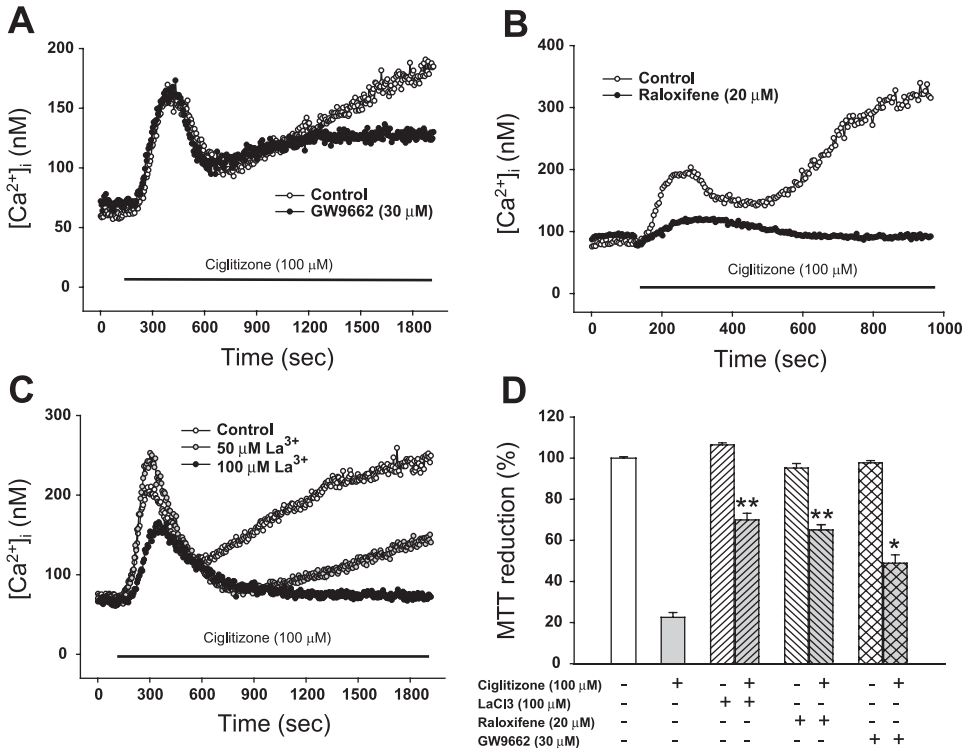

Fig. 3. Effects of antagonists for PPAR-γ, estrogen receptor, and store-operated Ca^{2+} channel on ciglitizone-induced secondary $[Ca^{2+}]_i$ increases and cell proliferation in primary cultured human uterine leiomyoma. Ciglitizone (100 μM)-induced intracellular Ca^{2+} measurements were performed in 1.8 mM Ca^{2+}-containing saline solution without (control) and with application of antagonists for PPAR-γ (GW9662; *A*), estrogen receptor (raloxifene; *B*) and store-operated Ca^{2+} channel (lanthanum chloride; *C*). These data represent the means of values measured in 30–40 cells in 3 separate experiments. *D*: cell proliferation was analyzed with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay at 48 h after treatment of ciglitizone and/or antagonists. Control cells were treated with vehicle only. Data are mean ± SE values from 3 separate experiments. *$P < 0.001$ vs. treatment with ciglitizone alone.

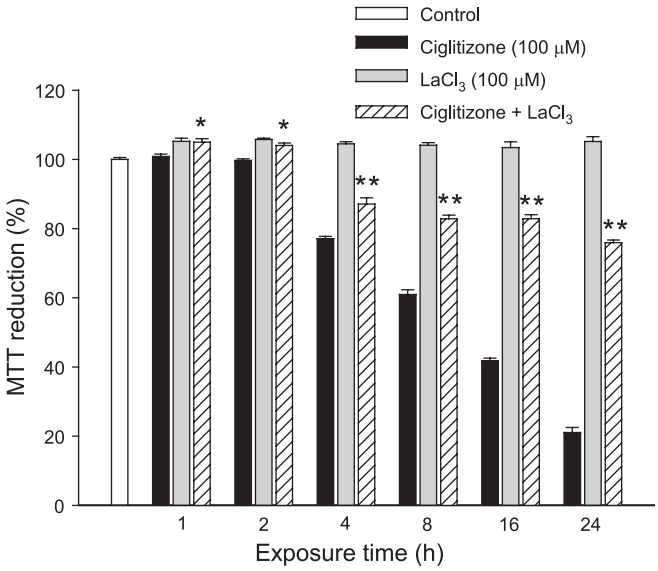

Fig. 4. Time course of ciglitizone and effects of lanthanum chloride on cell proliferation in primary cultured human uterine leiomyoma. Cell viability was analyzed with MTT reduction assay at the time periods indicated. Control cells were treated with vehicle only. Data are mean ± SE values from 3 separate experiments. *$P < 0.01$, **$P < 0.001$ vs. treatment with ciglitizone alone.

tion by using caffeine (10 mM) and KCl (25 mM), respectively (Fig. 5*B*). Caffeine and/or KCl significantly decreased the cell proliferation of uterine leiomyoma compared with that of controls ($P < 0.001$).

DISCUSSION

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

The two primary pathways for obtaining stimulus-increased levels of $[Ca^{2+}]_i$ in smooth muscle cells are release from sarcoplasmic reticulum stores and an influx from extracellular space (23). The increased $[Ca^{2+}]_i$ levels observed in myometrium and uterine leiomyoma could be from the sarcoplasmic reticulum. Release of Ca^{2+} from intracellular stores into the cytoplasm occurs through two classes of Ca^{2+} release channels, IP_3 receptors and ryanodine receptors, which may or may not coexist, depending on the tissue (5). Several mechanisms of intracellular Ca^{2+} mobilization are present in uterine myometrium. However, in human uterine myometrium only IP_3-induced 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 Ca^{2+} regulation (10, 34). Although IP_3-mediated Ca^{2+} release by a contractant such as oxytocin is a popular mechanism for increasing intracellular Ca^{2+} in myometrium (27), this type of Ca^{2+} release was not involved in this ciglitizone-induced $[Ca^{2+}]_i$ experiment. This study observed that the ryanodine receptor blocker dantrolene sodium completely suppressed the ciglitizone-induced initial $[Ca^{2+}]_i$ increase, whereas the IP_3-receptor blocker U-73122 did not have an effect on the $[Ca^{2+}]_i$ response. These results suggested that the pathways of ciglitizone-induced initial $[Ca^{2+}]_i$ increase are the ryanodine receptors of the sarcoplasmic reticulum in human uterine myometrium and leiomyoma.

In this study, the initial $[Ca^{2+}]_i$ increase induced by ciglitizone was inhibited by raloxifene, but the secondary $[Ca^{2+}]_i$ increase was attenuated by the PPAR-γ antagonists. These findings suggest that ciglitizone induced a rapid initial $[Ca^{2+}]_i$ 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-γ 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-γ and the estrogen receptor. The present study also showed that ciglitizone induced the initial $[Ca^{2+}]_i$ release from the sarcoplasmic reticulum through estrogen receptors in uterine leiomyoma, as shown in Fig. 3*B*.

Ciglitizone-induced SOCC activation in human uterine leiomyoma. Despite the same initial $[Ca^{2+}]_i$ increase in both types of cells, the ciglitizone-induced secondary $[Ca^{2+}]_i$ in-

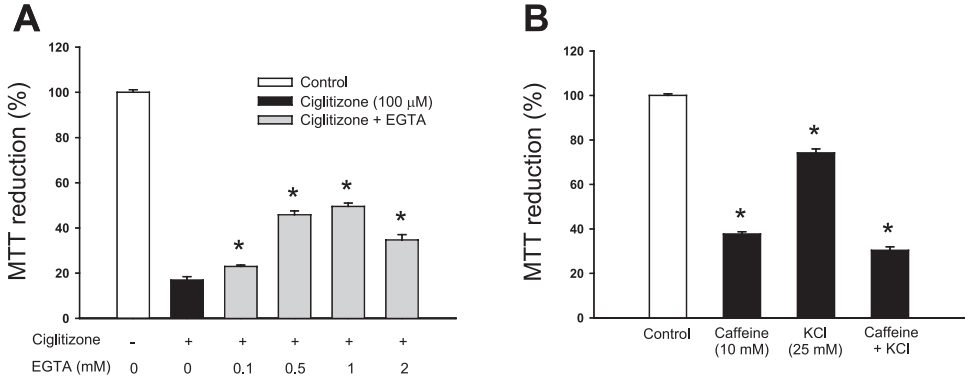

Fig. 5. Effects of extracellular and intracellular $[Ca^{2+}]$ on cell viability in primary cultured human uterine leiomyoma. *A*: extracellular free Ca^{2+} levels were reduced by increasing concentrations of EGTA, a chelating agent for extracellular calcium. *$P < 0.001$ vs. treatment with ciglitizone alone. *B*: intracellular Ca^{2+} levels were increased by caffeine, an agonist for ryanodine receptor, and KCl, an activator of Ca^{2+} influx. *$P < 0.001$ vs. control cells. Cell proliferation was analyzed with MTT reduction assay at 48 h after treatment of agents. Control cells were treated with vehicle only. Data are mean ± SE values from 3 separate experiments.

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, ciglitizone 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 ciglitizone-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.

Ciglitizone-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 signal transduction, gene expression, cell proliferation and apoptosis, and muscle excitation-contraction coupling (8, 20). At the cellular level, Ca^{2+} can be either a life signal or a death signal, because changes in cytosolic free Ca^{2+} level and Ca^{2+} content of intracellular stores can control cell growth and proliferation or induce programmed cell death (20).

Sustained increases of intracellular Ca^{2+} to the micromolar range are obviously deleterious to the signaling function of Ca^{2+} (8). In this study, the sustained increase in uterine leiomyoma of $[Ca^{2+}]_i$ could be expected for a long time via an activation of SOCC entry. Growing evidence suggests that Ca^{2+} plays a pivotal role in apoptosis, which is related to an activation of SOCCs (20, 22). The panoramic picture of Ca^{2+} signaling in apoptosis consists of Ca^{2+} release from sarcoplasmic or endoplasmic reticulum, depletion of intracellular Ca^{2+} stores, and subsequent activation of SOCC entry. Under these conditions, there would be two main consequences for the cell. First, the depletion of Ca^{2+} 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 Ca^{2+}, 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 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 ciglitizone-induced secondary $[Ca^{2+}]_i$ increase via activation of SOCC and recovered the inhibition of cell proliferation compared with treatment with ciglitizone alone. Therefore, ciglitizone-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-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 $[Ca^{2+}]_i$ through the activation of SOCCs, especially in human uterine leiomyoma.

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