Dual repressive effect of angiotensin II-type 1 receptor blocker telmisartan on angiotensin II-induced and estradiol-induced uterine leiomyoma cell proliferation

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BACKGROUND: Although uterine leiomyomas or fibroids are the most common gynecological benign tumor and greatly affect reproductive health and well-being, the pathophysiology and epidemiology of uterine leiomyomas are poorly understood. Elevated blood pressure has an independent, positive association with risk for clinically detected uterine leiomyoma. Angiotensin II (Ang II) is a key biological peptide in the renin-angiotensin system that regulates blood pressure. METHODS: In this study, we investigated the potential role of Ang II (1–1000 nM) in the proliferation of rat ELT-3 leiomyoma cells in vitro. RT–PCR and western blot analysis with cell proliferation and DNA transfection assays were performed to determine the mechanism of action of Ang II. RESULTS: Ang II induced ELT-3 leiomyoma cell proliferation (P < 0.01) and the expression of Ang II type 1 receptor (AT1R) and AT2R mRNA and protein was confirmed. Regarding the intracellular signaling pathway, the Ang II-induced cell proliferation was AT1R-, epidermal growth factor receptor-, extracellular-regulated kinase- and protein kinase C-dependent but was not dependent on the AT2R or phosphatidylinositol-3 kinase or JAK kinase. The AT1R blocker telmisartan, effectively repressed Ang II-induced and estradiol-induced cell proliferation (P < 0.01). AT1R, but not AT2R, plays a role in Ang II-induced ELT-3 cell proliferation. CONCLUSIONS: These experimental findings in vitro highlight the potential role of Ang II in the proliferation of leiomyoma cells.

Introduction

Uterine leiomyomas or fibroids are the most common gynecological benign myometrial neoplasms, occurring with an incidence as high as 77% in reproductive age women (Cramer and Patel, 1990). These tumors are a major indication for hysterectomy in premenopausal women and annually account for 33% of all hysterectomies (over 200 000) in the USA (Wilcox et al., 1994; Lepine et al., 1997). Although these benign tumors represent a significant health concern for women, the causes of uterine leiomyomas are unknown (Walker and Stewart, 2005). Epidemiological and experimental evidence has established an essential role of ovarian hormones in the pathogenesis of this disease. Numerous studies have shown that the growth of leiomyomas depends on the ovarian hormones estrogen and progesterone (Rein, 2000). Risk factors include age, African-American ethnicity, nulliparity and obesity (Flake et al., 2003). It has also been reported that elevated blood pressure has an independent, positive association with risk for clinically detected uterine leiomyoma among premenopausal women (Boynton-Jarrett et al., 2005). However, the underlying mechanism of this association remains unknown.

There is increasing evidence that Angiotensin II (Ang II), a major regulator of blood pressure and cardiovascular homeostasis, is involved in the regulation of cell proliferation, angiogenesis, inflammation and tissue remodeling (Deshayes and Nahmias, 2005). Many of these studies suggested the existence of local actions of Ang II that appear to be different from the conventional circulating Ang II. Two subtypes of Ang II receptors, type 1 (AT1R) and type 2 (AT2R), have been identified. Most physiological effects of Ang II have been attributed to stimulation of the AT1R (Deshayes and Nahmias, 2005). AT1R blockers (ARBs) are widely used in the treatment of...
hypertension and hypertension-related cardiovascular end-organ damage (de Gasparo et al., 2000).

It is well established that AT1R induces cell proliferation in a variety of cellular models, including vascular smooth muscle cells (VSMCs) and cancer cells, by activating various intracellular cascades of protein kinases usually associated with growth factor stimulation (Deshayes and Nahmias, 2005). Most notably, AT1R transactivates the epidermal growth factor receptor (EGFR) in prostate (Uemura et al., 2003) and breast cancer (Greco et al., 2003) cells, leading to extracellular-regulated kinase (ERK) activation, phosphorylation of signal transducer and activator of transcription 3 (STAT3) and activation of protein kinase C (PKC) (Deshayes and Nahmias, 2005).

The Eker rat is an animal model for spontaneous uterine leiomyoma. Eker rat leiomyomas are histologically similar to human leiomyomas and display smooth muscle markers (Everitt et al., 1995). Cell lines derived from these tumors (ELT-3 cells) express estrogen receptor (ER) and progesterone receptor (PR) and have been successfully used in many studies to investigate the hormonal modulation of leiomyomas (Howe et al., 1995a,b). It has been reported that human leiomyoma cell cultures undergo a 75% decrease in ER and PR expression within 8 h of primary culture and hormone responsiveness is consistently lost (Severino et al., 1996), making the use of such cultures for in vitro studies problematic. As an alternative, rat ELT-3 cells are a unique and powerful tool to study the effect of steroid hormones and steroid receptor signaling in leiomyoma cells.

In this study, we examined whether Ang II induced the proliferation of rat uterine leiomyoma ELT-3 cells and analysed the mechanism of Ang II action.

Materials and Methods

Chemical compounds

Ang II, 17β estradiol (E2), wortmannin [phosphoinositide-3 kinase (PI3K) inhibitor], PD123319 (AT,R antagonist) and bisphenol A diglycidyl ether (BADGE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Telmisartan (AT,R blocker) was a gift from Boehringer Ingelheim, Biberach an der Riss, Germany. 15-deoxy-delta(12, 14)-prostaglandin J2 (15d-PGJ2) and Iosartan (AT,R blocker) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). AG1478 (specific inhibitor of the tyrosine kinase activity of EGFR) was purchased from Calbiochem (Darmstadt, Germany). PD98059 [mitogen-activated protein kinase (MEK I) inhibitor], AG490 (JAK2 inhibitor) and staurosporine (PKC inhibitor) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). E2 and BADGE were dissolved in ethanol (EtOH) and the inhibitors were dissolved in dimethylsulfoxide (DMSO). The resultant stock solutions (0.1% vol/vol) were added to the culture medium at the concentrations indicated in the Results section. Control cells were treated with an equal amount of EtOH (0.1% vol/vol) or DMSO (0.1% vol/vol) to control for any cytotoxic effects of EtOH or DMSO themselves.

Cell culture

The Eker rat-derived uterine leiomyoma cell line ELT-3 cells were cultured in 5% CO2 at 37°C in DF8 medium containing 10% fetal calf serum (FCS) as previously described (Howe et al., 1995a,b). Serum-free, phenol red-free DF8 basal medium containing 1% bovine serum albumin (Sigma-Aldrich) was used to treat ELT-3 cells with test compounds.

RNA extraction and RT–PCR

Total cellular RNA was extracted from cultured cells using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer’s protocol. First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. AT1R and AT2R were amplified using the following primer pairs: 5′-CAATCTCGCCCTGGCTGACTT-3′ (AT1R forward), 5′-TCTTTCTCAGAGTGTATGTC-3′ (AT1R reverse), 5′-AAGTGTGATTGGGAG-3′ (AT2R forward) and 5′-TTCAGGTCAGAAAAGACC-3′ (AT2R reverse) as previously described (Xiao et al., 2004; Juan et al., 2005). After denaturation at 95°C for 5 min, 1-μl aliquots of the cDNA in a 50 μl reaction mixture were amplified with 25 pmol each of sense and antisense primers and Taq PCR Master Mix (Qiagen). Reactions for AT1R amplification were performed for 45 s at 94°C, 1 min at 54°C and 1 min at 72°C for 35 cycles with a final extension for 7 min at 72°C. Reactions for AT2R amplification were performed for 35 s at 94°C, 1 min at 55°C and 1 min at 72°C for 35 cycles with a final extension for 7 min at 72°C. The amplified mixture (10 μl) was analysed by 2% agarose gel electrophoresis and the DNA bands were visualized by staining with ethidium bromide.

SDS-PAGE and western blot analysis

Cells were harvested and lysed for 30 min in 1 ml of lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM NaF, 0.75 mM phenylmethylsulphonyl fluoride, 15% glycerol and 10 μg/ml each of aprotinin and leupeptin] as previously described (Takeda et al., 1997). Samples (60 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% gel and analysed by blotting with rabbit polyclonal anti-AT1 antibody (Alfa Diagnostic International, San Antonio, TX, USA) or rabbit polyclonal anti-AT2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The proteins were detected using enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer’s instructions.

Assessment of cell proliferation

Cell proliferation was measured using the cell counting or the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulphophenyl)-2H-tetrazolium] assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA).

Cell counting

ELT-3 cells were plated at 2500 cells/well in 24-well plates in DF8 medium supplemented with 10% FCS and allowed to attach overnight. After 24 h of starvation in DF8 basal medium, the cells were treated on Day 0 with medium containing the appropriate test compounds, and then further incubated for appropriate periods. In the experiments examining the effects of Ang II on cell growth kinetics, we replaced the medium supplemented with Ang II every 3 days. The cells were washed once with 1x phosphate-buffered saline and then collected using trypsin/EDTA and counted on Day 1, 3, 5 and 7 in a hemocytometer using light microscopy. Three wells were counted for each condition. All experiments were repeated at least three times.
**MTS assay**
The cells were placed in DF8 medium with 10% FCS at a concentration of 1000 cells/well in 96-well plates for 24 h. Subsequently, the cells were incubated in DF8 basal medium for another 24 h. Ang II was added at the concentrations indicated in the Results section and the cells were further cultured for 3 days. Then the MTS assay was performed as recommended by the manufacturer. The amount of formazan product is proportional to the metabolic activity of cells and provides a measurement of cell growth. All experiments were performed in sextuplicate and repeated at least three times with similar results. Results are expressed as the light absorbance at 490 nm.

**DNA transfection and luciferase assays**
ELT-3 cells (1.5 x 10^6 cells/well) were seeded in 24-well culture plates. Twenty-four hours after seeding, 0.36 μg of peroxisome proliferator-activated receptor response element (PPRE)-luciferase reporter plasmid (a gift from Dr. R. Evans) and 0.04 μg of pSV40 LacZ (as an internal control for transfection efficiency) were transiently transfected into ELT-3 cells using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were treated with an appropriate concentration of 15d-PGJ2 or ARBs. Twenty-four hour later, cells were lysed in lysis buffer. Cell extracts were prepared and assayed for luciferase activity using the Promega Luciferase Assay System (Promega) and for β-galactosidase activity using Galacto-Light Plus (Applied BioSystems, Foster City, CA, USA) according to the manufacturer’s instructions. The values of luciferase activity were normalized with β-galactosidase values to correct for differences caused by unequal transfection efficiency. All experiments were performed in triplicate and repeated at least three times with essentially similar results. Results are expressed as relative luciferase activity.

**Statistical analysis**
Statistical analysis was performed using Excel 2003 (Microsoft, USA) with the add-in software Statcel 2 (OMS, Tokyo, Japan). The significance of differences between means was evaluated by Student’s t-test and analysis of variance, followed by Scheffe’s F-test. All data are expressed as mean ± SD. Statistical significance was set at P < 0.05.

**Results**

**Expression of AT1 and AT2 receptor in ELT-3 leiomyoma cells**
It has been reported that Ang II receptors (AT1R and AT2R) are expressed in human leiomyoma cells (Matsumoto et al., 1996). To investigate whether AT1R and AT2R were expressed in ELT-3 cells, the total cellular RNA from ELT-3 cells was subjected to RT–PCR using primers for AT1R and AT2R (Fig. 1A). RT–PCR products with the expected size for AT1R (1036 bp) and AT2R (416 bp) were detected. To further investigate whether the Ang II receptors are evident at the protein level, whole cell lysate from ELT-3 cells was analysed by western blotting with specific antibody for AT1R and AT2R (Fig. 1B). We could detect the expected band for AT1R (45 kDa) and AT2R (44 kDa).

**Ang II-induced proliferation of ELT-3 cells**
Next we analysed whether Ang II could stimulate the proliferation of ELT-3 cells. ELT-3 cells were incubated with various concentrations of Ang II for 3 days and the cell number was counted (Fig. 2A). We found that treatment with Ang II significantly increased the ELT-3 cell number compared with the control in a dose-dependent manner. To evaluate the cell growth reflecting the increasing metabolic activity of cell, we analysed Ang II-induced ELT-3 cell proliferation using MTS assay (Fig. 2B). The number of cells, as reflected by increased formazan generation, increased in cultures treated with Ang II in a dose-dependent manner. We next determined the response of cell growth kinetics to Ang II. The cell proliferation of ELT-3 cells with or without Ang II (100 nM) was examined for up to 7 days. The cells increased in number with or without Ang II during the period of the study. Treatment with Ang II resulted in an increase in cell number compared with the number of control cells (Fig. 2C). Cell morphology, trypan blue exclusion dye testing and cells undergoing apoptosis detected by the uptake of a purple dye (APOPercentage Apoptotic Assay, Biocolor, Belfast, Northern Ireland) were similar between the control and Ang II-treated groups (data not shown). These results show that the growth effects of Ang II were mediated through the enhancement of proliferation, not through decreased apoptosis.

**Signal transduction pathways of Ang II-induced ELT-3 cell proliferation**
Most physiological effects of Ang II are mediated through the action of AT1R. To examine the involvement of AT1R in Ang
results.

more than three independent experiments were done with similar triplicate samples of a representative experiment; as for Figs 3–5, AT1R in cell proliferation have been elucidated in a variety of cellular models which transactivate EGFR signaling. We analysed the effect of a specific inhibitor of the tyrosine kinase activity of EGFR (AG1478) on the Ang II-induced proliferation of ELT-3 cells (Fig. 3D). AG1478 strongly inhibited the Ang II-induced cell proliferation. AG1478 also repressed the growth of cells in the absence of Ang II-stimulation (Fig. 3I). These results indicate that EGFR-mediated signaling is the major pathway of Ang II-induced ELT-3 cell proliferation and this pathway is also functioning without Ang II stimulation.

EGFR signaling is reported to lead to ERK, PKC, PI3K and STAT3 activation (Deshayes and Nahmias, 2005). To investigate the involvement of signaling via these molecules in Ang II-induced cell proliferation, we examined the effect of PD98059 (MEK I inhibitor) (Fig. 3E), staurosporine (PKC inhibitor) (Fig. 3F), wortmannin (PI3K inhibitor) (Fig. 3G) and AG490 (JAK2 inhibitor) (Fig. 3H). PD98059 and staurosporine inhibited the Ang II-induced cell proliferation effectively. The growth of cells treated with PD98059 alone and staurosporine alone did not change compared with the growth of control cells (data not shown). On the other hand, wortmannin and AG490 did not affect the Ang II-induced cell proliferation. These results indicate that Ang II promotes ELT-3 cell proliferation through EGFR, MEK-ERK or PKC signaling.

**Additive effect of E2 on Ang II-induced ELT-3 cell proliferation**

It was previously reported that E2 antagonizes the AT1-R-induced cell proliferation of VSMCs (Takeda-Matsubara et al., 2002). Next we analysed the effect of E2 treatment on the Ang II-induced ELT-3 cell proliferation (Fig. 4). E2 treatment (10 nM) significantly increased the ELT-3 cell number as effectively as Ang II treatment. E2 combined with Ang II resulted in cell counts that were additive. These results suggested that E2 has diverse growth effects on Ang II-induced cell proliferation depending on the cell type.

**Telmisartan represses the E2-induced proliferation of ELT-3 cells**

It has been reported that peroxisome proliferator-activated receptor (PPAR)γ activation has a growth inhibitory effect on uterine leiomyoma cells and this inhibition is mediated by negative cross-talk between the ER and PPAR signaling pathways (Houston et al., 2003). Telmisartan has been shown to induce PPARγ activity independent of the AT1R (Schupp et al., 2004). We examined whether telmisartan functions as a PPARγ ligand and inhibits the E2-induced proliferation of ELT-3 cells. Telmisartan enhanced the transcription of a luciferase reporter driven by a PPRE-containing promoter, but losartan did not (Fig. 5A). E2-induced ELT-3 cell proliferation was significantly inhibited by telmisartan, but not by losartan (Fig. 5B). This inhibitory effect was reduced by BADGE (a PPARγ antagonist) in a dose-dependent manner, and treatment with 10 μM BADGE completely restored the E2-induced proliferation (Fig. 5C). These results indicate that telmisartan inhibits the E2-induced proliferation of ELT-3 cells by acting as a PPARγ ligand.
Discussion

This is the first report about Ang II-induced leiomyoma cell proliferation. In this work, we have shown that (i) AT$_1$R and AT$_2$R are expressed in ELT-3 leiomyoma cells, (ii) Ang II-induced leiomyoma cell proliferation is mediated through AT$_1$R and EGFR signaling, (iii) MEK-ERK and PKC pathways, but not PI-3 kinase or JAK kinase pathways, are involved and (iv) telmisartan inhibits both Ang II-induced and E$_2$-induced ELT-3 cell proliferation.

Hypertension has been reported to be associated with increased risk of leiomyomata (Faerstein et al., 2001; Luoto et al., 2001). Moreover, a recent prospective study showed
that elevated blood pressure has an independent, positive association with risk for clinically detected uterine leiomyoma among premenopausal woman (Boynton-Jarrett et al., 2005). Elevated blood pressure may cause smooth muscle cell injury and cytokine release and increase the risk of leiomyomata, in a process analogous to athelosclerotic changes in arterial smooth muscle cells. In addition to causing vasoconstriction, Ang II affects the transcription of multiple genes concerned with cell proliferation, angiogenesis, inflammation and atherogenesis or thrombus formation (Faerstein et al., 2001; Boynton-Jarrett et al., 2005). Ang II-induced leiomyoma cell proliferation may play a crucial role in the association of hypertension and uterine leiomyoma.

Two subtypes of Ang II receptors, AT₁R and AT₂R, have been reported to be expressed in human uterine leiomyoma (Matsumoto et al., 1996) and our data also showed that the two Ang II receptors are present in ELT-3 cells. Telmisartan and losartan repressed Ang II-induced cell proliferation, but AT₂ antagonist (PD123319) did not affect this proliferation. We have also studied the effect of AT₂ agonist (CGP42112) on the proliferation of ELT-3 cells and CGP42112 had no effect on proliferation (our unpublished data). These results showed that AT₁R plays a major role in Ang II-induced ELT-3 cell proliferation.

The Ang II-AT₁R signaling pathway has been reported to play an important role in the regulation of cancer cell proliferation. Transactivation of EGFR has emerged as a central mechanism by which AT₁R stimulates the proliferation of cancer cells and leads to ERK activation, STAT3 phosphorylation and PKC activation (Deshayes and Nahmias, 2005). Our data showed that AngII promotes leiomyoma cell proliferation through EGFR, MEK-ERK or PKC signaling. AT₁R-mediated transactivation of EGFR, such as seen in cancer cell proliferation, seemed also to be present in leiomyoma cells. Since the EGF-EGFR signaling pathway has been shown to play a crucial role in regulating leiomyoma growth (Harrison-Woolrych et al., 1994; Shimomura et al., 1998), EGFR transactivated by Ang II-AT₁R signaling probably uses the same signaling pathway.

Cross-talk between the signal transduction pathways of Ang II and E₂ has been reported in other cells (Takeda-Matsubara et al., 2002). Though in our study E₂ treatment had an additive effect on Ang II-induced cell proliferation in ELT-3 cells, a previous study showed that E₂ antagonizes AT₁R-induced cell proliferation of VSMCs (Takeda-Matsubara et al., 2002). Two diverse growth responses of E₂, an inhibitory effect on VSMCs and a mitogenic effect on ELT-3 cells, have also been reported (Finlay et al., 2003). The mechanism of this

**Figure 4:** Additive effect of E₂ on Ang II-induced cell proliferation. Control cells were treated with EtOH (0.1% vol/vol). The cells were treated with Ang II (100 nM), E₂ (10 nM) or both for 3 days. Cells were counted as described in Materials and Methods. The values are mean ± SD of triplicate samples of a representative experiment. Different superscripts indicate statistically significant differences (P < 0.05).

**Figure 5:** The inhibitory effect of telmisartan, an AT₁R blocker, on E₂-induced ELT-3 cell proliferation. (A) Transactivation of PPRE in ELT-3 cells by telmisartan. Luciferase activity was assayed in ELT-3 cells transfected with PPRE-luciferase reporter construct and further incubated with PPARγ ligand 15d-PGJ2 (PG) (10 μM), telmisartan (Tel; 10 μM) or losartan (Los; 10 μM) for 24 h. The luciferase activity of the control cells was defined as 1. *P < 0.05; **P < 0.01 versus control. (B) Control cells (open bars) were treated with EtOH (0.1% vol/vol) and DMSO (0.1% vol/vol). The cells were incubated with or without telmisartan (Tel; 10 μM) or losartan (Los; 10 μM) in the presence of E₂ (10 nM) for 3 days. Cell numbers were counted as described in Material and Methods. Data are expressed as the percentage of untreated control. **P < 0.01 versus without inhibitor. (C) PPARγ antagonist reduced the inhibitory effect of telmisartan. The cells were incubated with or without telmisartan (Tel; 10 μM) and 1 or 10 μM BADGE in the presence of E₂ (10 nM) for 3 days. Cell numbers were counted as described in Materials and Methods.
The expression of TSC2 may also explain the different effect of E₂ on the Ang II-induced cell proliferation.

ARBs have metabolic actions and lower the risk for type 2 diabetes through the activation of PPARγ (Dahlof et al., 2002; Picard and Auwerx, 2002). Among various ARBs, telmisartan is the most potent and stimulates PPARγ at pharmacologically relevant concentrations (Kurtz et al., 2004; Schupp et al., 2004). In this study, we used telmisartan at concentrations similar to those shown to be active in pharmacological studies and showed that telmisartan inhibits not only the Ang II-induced ELT-3 cell proliferation but also the E₂-induced proliferation by acting as a PPARγ ligand.

The limitation of this study is that these findings were based on in vitro experiments and there is no data about the clinical relevance of Ang II and human leiomyoma growth. It would be useful to study the change of fibroid size in hypertensive women with leiomyoma treated with ARBs compared with untreated women. Further examination of the mechanism of the Ang II-induced proliferation of ELT-3 cells will provide new insights into the regulation of leiomyoma cell proliferation. We are now trying to study the effect of telmisartan using the Eker rat uterine leiomyoma in vivo model.

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References


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