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Effects of PPARγ Agonists on the Expression of Leptin and Vascular Endothelial Growth Factor in Breast Cancer Cells

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The obesity hormone leptin has been implicated in breast cancer development. Breast cancer cells express the leptin receptor and are able to synthesize leptin in response to obesity-related stimuli. Furthermore, leptin is a positive regulator of vascular endothelial growth factor (VEGF) and high levels of both proteins are associated with worse prognosis in breast cancer patients. Peroxisome proliferator-activated receptor γ (PPARγ) ligands are therapeutic agents used in patient with Type 2 diabetes and obesity which have recently been studied for their potential anti-tumor effect. Here, we studied if these compounds, ciglitazone and GW1929, can affect the expression of leptin and VEGF in breast cancer cells. In MDA-MB-231 and MCF-7 breast cancer cells, treatment with submolar concentrations of ciglitazone and GW1929 elevated the expression of leptin and VEGF mRNA and protein, and increased cell viability and migration. These effects coincided with increased recruitment of PPARγ to the proximal leptin promoter and decreased association of a transcriptional factor Sp1 with this DNA region.

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Leptin is a hormone produced mostly by the adipose tissue. In addition to its well-documented role in the control of appetite and energy homeostasis, leptin is known to regulate various physiological and pathological processes in the peripheral organs. The accumulating evidence suggests that leptin can induce growth and progression of different cancer types, which in part could explain the link between excess body adipocity and increased cancer risk (Garofalo and Surmacz, 2006). In fact, data obtained in cellular and animal cancer models demonstrated that leptin can act as a mitogenic, motogenic, survival and angiogenic factor (Surmacz, 2007; Anagnostoulis et al., 2008; Ferla et al., 2011).

The importance of leptin signaling in breast cancer is confirmed by the fact that malignant breast tumors, but not normal mammary epithelium, overexpress both leptin and ObR (Garofalo et al., 2006; Fiorio et al., 2008) and the leptin/ObR system often correlates with higher tumor grade and worse prognosis (Garofalo et al., 2006).

The mechanism of leptin expression in breast cancer cells has been partially elucidated. We demonstrated that leptin can be induced by hyperinsulinemia and hypoxia (Garofalo et al., 2006; Koda et al., 2007; Cascio et al., 2008). On the molecular level, these effects are mediated through interactions of Sp1 and/or hypoxia-inducible factor-1 alpha (HIF-1α) with specific motifs within the leptin gene promoter (Bartella et al., 2008; Cascio et al., 2008). In addition, leptin expression in breast cancer cells is likely to be modulated by other transcription factors (Bartella et al., 2008), for example, peroxisome proliferator-activated receptor γ (PPARγ; Hollenberg et al., 1997).

The actions of leptin are mediated through the leptin receptor (ObR). Binding of leptin to the long form of ObR induces multiple pathways, including the Jak/STAT3, ERK1/2, and PI-3K/Akt pathways. In addition, leptin induces cyclin D1 expression and retinoblastoma protein hyperphosphorylation (Garofalo and Surmacz, 2006). Leptin also influences other signaling systems, for example, it can transactivate the epidermal growth factor receptor 1 (EGFR1), the human epidermal growth factor receptor 2 (HER2), and the insulin-like growth factor 1 receptor (IGF-IR; Shida et al., 2005; Fiorio et al., 2008; Ozbay and Nahta, 2008). Furthermore, leptin can induce the synthesis of fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF; Cao et al., 2001; Garofalo and Surmacz, 2006; Surmacz, 2007). Inhibition of leptin signaling suppresses the expression of VEGF/VEGFR2 and reduces the growth of mouse mammary tumors and human breast cancer xenografts (Gonzalez et al., 2006; Otvos et al., 2011).

VEGF promotes growth, proliferation, migration, formation of new blood vessels (Bernatchez et al., 2002) and survival of endothelial cells. VEGF regulation can occur at both transcriptional and post-transcriptional levels in a cell-specific manner (Claffey and Robinson, 1996). The proximal GC-rich region in the VEGF promoter contains binding sites for AP2, Erg-1, WT1, NFκB, and SP1/SP3 transcription factors, while

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Conflict of interest: nothing to declare.

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distal enhancer sites bind HIF-1α (Shima et al., 1996; Gille et al., 1997; Hanson et al., 2007). In mouse mammary tumor cells, 4T1 leptin upregulates VEGF expression through Sp1 (Gonzalez-Perez et al., 2010).

PPARγ are ligand-activated transcription factors, members of the nuclear receptor superfamily. PPARγ can be activated by its natural ligand, the prostanoid 15-deoxy-prostaglandin J2, its synthetic ligands, such as the *N*-aryl tyrosine derivative as GW1929 as well as thiazolidinediones (TZDs), including troglitazone, rosiglitazone, pioglitazone, and ciglitazone, a class of drugs used to improve lipid and glucose metabolism in Type 2 diabetes (Saltiel and Olefsky, 1996). In rats, treatment with PPARγ synthetic ligands reduced leptin mRNA levels in the adipose tissue (Kallen and Lazar, 1996), and results obtained in primary rat adipocytes demonstrated that the proximal leptin promoter (−65 to +9) is negatively regulated by PPARγ ligand. In this case, PPARγ does not bind directly any specific DNA sequence within this region, but antagonizes the association of a transcriptional leptin regulator, C/EBPα, at −65/+9 (Hollenberg et al., 1997). In obese humans, 200 mg troglitazone administered twice daily does not affect fasting plasma leptin concentrations (Nolan et al., 1996), and in Type 2 diabetic patients, leptin levels do not change following treatment with rosiglitazone (8 mg/day) and pioglitazone (45 mg/day; Miyazaki and DeFronzo, 2008). In human cultured adipocytes, activation of PPARγ alone had little effect on leptin production, but inhibited dexamethasone-induced leptin release (Williams et al., 2000).

There is evidence that the expression of PPARγ is increased in several cancers, including breast, prostate, pancreas, and colon (Koeffler, 2003; Kristiansen et al., 2006) and decreased in others, such as esophagus and uterus (Terashita et al., 2002; Jung et al., 2005). However, the role of PPARγ in cancer progression remains controversial. While some reports demonstrated anti-proliferative role of PPARγ ligands in cellular and animal models of human cancer (Grommes et al., 2004), other documented that activation of PPARγ can induce cell growth and tumor proliferation, depending on the dose and duration of treatment (Clay et al., 2001; Fujita et al., 2003; Choi et al., 2008; Talbert et al., 2008). Notably, PPARγ agonists have been shown to stimulate angiogenesis through upregulation of VEGF and other yet unidentified pro-angiogenic factors (Biscetti et al., 2008). Recently, the Food and Drug Administration (FDA) announced that use of pioglitazone for more than 1 year may be associated with an increased risk of bladder cancer (Lewis et al., 2011). In this regard, several studies in the last years seem to confirm the correlation between the use of TZDs and the risk of bladder cancer among people with Type 2 diabetes (Colmers et al., 2012; Kermode-Scott, 2012; Mamtani et al., 2012).

The role of PPARγ in breast cancer is not clear. In vivo and in vitro data demonstrate that PPARγ ligands can suppress breast cancer growth (Elstner et al., 1998; Suh et al., 1999). However, experiments using transgenic mice suggest that constitutive PPARγ signaling has no effects on mammary gland differentiation or function, but accelerates tumor appearance and mortality in mammary cancer-prone mice expressing the polyoma middle T antigen (Saez et al., 2004). In humans, troglitazone does not exhibit clinical response in treatment-refractory metastatic breast cancer (Burstein et al., 2003). Similarly, rosiglitazone has no effect on cell proliferation and fasting serum leptin levels in a prospective study evaluating therapy of early-stage breast cancer (Yee et al., 2007).

In light of the ambiguous role of PPARγ in cancer and considering that high levels of leptin and VEGF are strongly linked to worse prognosis of breast cancer, we investigated if and how PPARγ agonists can regulate leptin and VEGF expression in breast cancer cells.

Materials and Methods

PPARγ ligands

Ciglitazone (CGZ) and GW1929 (GW) were used to specifically activate, while T0070907 (T0) to inhibit PPARγ. All compounds were purchased from Sigma–Aldrich (St. Louis, MO) and solubilized in DMSO.

Cell culture

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM:F12; Cellgro, Herndon, VA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Cellgro).

Cell growth assay

For cell growth assays, MDA-MB-231 and MCF-7 cells were plated into 96-well plates, incubated at 37°C in a humidified atmosphere of 5% of CO_2, allowed to attach overnight, transferred to SFM for 24 h and then stimulated with PPARγ agonists alone (CGZ or GW) or in combination with the antagonist (T0) at concentrations of 0.5, 5, and 10 μM/L for 24 h, or 0.05% dimethyl sulphoxide (DMSO; as control). After 24 h, cell proliferation was assessed by an MTT assay performed according to manufacturer's instructions (Millipore, Temecula, CA).

Real-time quantitative PCR (QRT-PCR)

MDA-MB-231 and MCF-7 were synchronized in SFM for 24 h, and then treated for 24 h with 0.5 μM of CGZ or 0.5 μM GW, alone or in combination with 0.5 μM T0.

Total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Five microgram of total RNA was reverse transcribed using the TaqMan RT kit (Applied Biosystems, Branchburg, CA), according to vendor's instructions. Five microliters of the RT products were used to amplify leptin and VEGF sequences using the Hs00174877 A1 Lep and the Hs00900054_m1 VEGFA TaqMan probe (Applied Biosystems). To normalize QRT-PCR reactions, parallel TaqMan β-actin control reagents assays (Applied Biosystems) were run on each sample. Changes in the leptin and VEGF mRNA content relative to β-actin mRNA were determined using a comparative CT method (ABI User Bulletin no. 2) to calculate changes in CT, and ultimately fold and percent change. An average CT value for each RNA was obtained for triplicate reactions.

ELISA

Subconfluent MDA-MB-231 and MCF-7 cell cultures were placed in SFM for 24 h, and then treated with 0.5 μM of CGZ or 0.5 μM GW for 24 h, alone or in combination with 0.5 μM T0. Conditioned medium obtained from four plates treated cell cultures was collected, centrifuged at 2,000 rpm for 5 min, and the supernatants frozen at −80°C until use, to evaluate the concentration of leptin. For the VEGF, instead, was used conditioned medium obtained from a single plate.

The abundance of leptin and VEGF in cell conditioned medium was measured using the VEGF ELISA (R&D Systems, Minneapolis, MN) and ELISA low range leptin kit (Arcus Biologicals, Modena, Italy) following manufacturer's instructions.

Chromatin immunoprecipitation (ChIP)

Following treatment with 0.5 μM of CGZ for 24 h, the cells were crosslinked with 1% formaldehyde and chromatin was collected and sonicated. Soluble chromatin was immunoprecipitated with the following antibodies (Abs): PPARγ (H-100) Ab (Santa Cruz Biotechnology, Santa Cruz, CA), Sp1 PEP_2 (Santa Cruz Biotechnology). DNA–protein immune complexes were eluted, reverse crosslinked, and DNA was extracted.

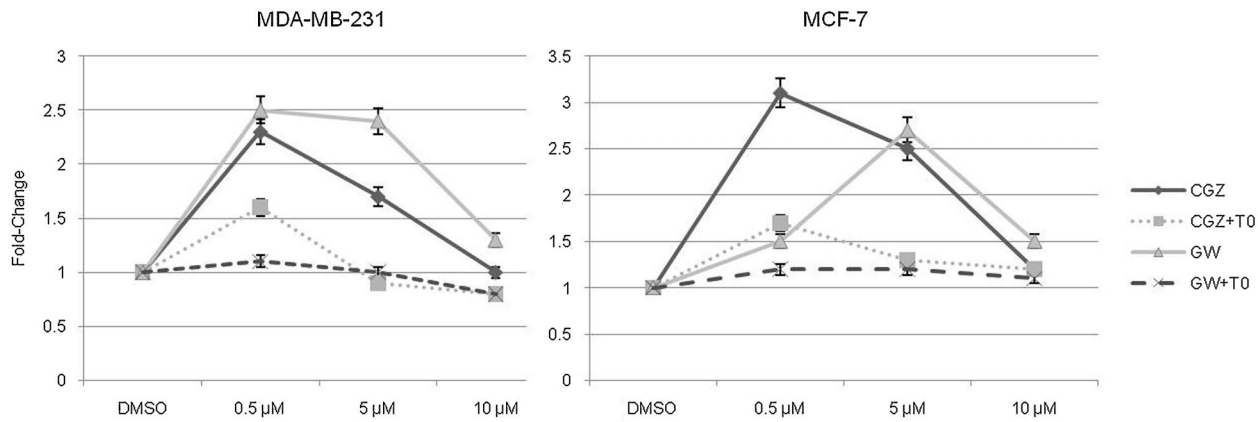

Fig. 1. Effects of different doses of PPARγ ligands on breast cancer cells growth. MTT analysis for MDA-MB-231 and MCF-7 cells stimulated with PPARγ agonists alone (CGZ or GW) or in combination with the antagonist (T0) at concentrations of 0.5, 5, and 10 μM for 24 h, compare to dimethyl sulphoxide (DMSO) as described in Materials and Methods Section. Data, representative of three different experiments each performed in triplicate, are presented as fold change ± of cell viability relative to the control ($P \leq 0.05$).

Real-time quantitative PCR (QRT-PCR) analysis of ChIP DNA fragments

QRT-PCR of DNA obtained from ChIP was performed using SYBR Green I (Applied Biosystems, Foster City, CA). The presence of leptin proximal promoter DNA in PPARγ and Sp1 ChIPs was tested using the following primers from −488 to +26: forward 5′-TCTAACCCTGGGCTTCCCT-3′ and reverse 5′-AACCGTTGGCGCTGCGATT-3′. The PCR conditions were: 40 cycles at 1 min, 30 sec at 95°C, 30 sec at 58°C, 1 min at 72°C. In control samples, the primary Abs were replaced with appropriate non-immune IgGs. To normalize QRT-PCR reactions, chromatin inputs were used as control and were run on each sample. Changes in the content relative to input were determined using a comparative CT method (ABI User Bulletin no. 2) to calculate changes in CT, and ultimately fold and percent change. An average CT value for each samples was obtained for triplicate reactions.

Western blotting (WB)

The expression of nuclear proteins was analyzed in 100 μg of nuclear cell lysates. The Abs were used for WB: PPARγ (H-100) Ab (Santa Cruz), Sp1 (PEP2) Ab (Santa Cruz), and nucleolin Ab (Santa Cruz).

Matrigel invasion assay

The invasive potential of MDA-MB-231 and MCF-7 breast cancer cells after treatment for 24 h with 0.5 μM CGZ was assessed using BioCoat invasion chambers (24-well companion plates with cell culture inserts containing 8-μm pore size filters coated with matrigel; Becton Dickinson Biosciences, Franklin Lakes, NJ), following manufacturer's instructions. 5×10^4 MDA-MB-231 and MCF-7 cells treated with DMSO or 0.5 μM/L of CGZ were suspended in 500 μl of DMEM and plated in upper chambers of inserts, while the chemoattractant (50% FBS) was placed in lower chambers. The chambers were incubated for 24 h at 37°C, 5% CO_2. After that, the non-invading cells were removed from the upper surface of the filter with a cotton-tipped applicator, while the cells that migrated through the pores and attached to the undersurface of the filter were fixed and stained. The membranes were mounted on glass slides, and the number of cells in five random microscopic fields (20× magnification) was scored for each experimental group. All experiments were run in triplicate, and invasiveness was calculated as the percentage invasion through the matrigel relative to the migration through the control membrane, as described in the manufacturer's instructions.

Results

Effects of PPARγ ligands on breast cancer cells viability

Considering the differential activity of PPARγ in cancer cells depending on doses applied and duration of treatment (Clay et al., 2001; Fujita et al., 2003; Choi et al., 2008; Talbert et al., 2008), we first tested the effects of different concentrations (0.5, 5, and 10 μM) of CGZ and GW on viability of MDA-MB-231 and MCF-7 breast cancer cells at 24 h. Each treatment was used alone or in combination with the antagonist T0 (Fig. 1 and Table 1).

The results of MTT assays suggested that in both cell lines the activation of PPARγ by lower concentrations (0.5 and 5 μM) of CGZ and GW increase cell viability relative to DMSO-treated controls. At higher concentrations (10 μM) CGZ and GW did not induce any significant change in cell viability versus control (Fig. 1; Table 1).

In parallel experiments, CGZ and GW were tested in combination with T0, a PPARγ antagonist. In both cell lines, T0 reduced the effects of agonists especially for concentrations 0.5 and 5 μM (Fig. 1; Table 1).

Effects of submolar concentrations of PPARγ agonists on leptin expression

In order to determine whether low concentrations of PPARγ agonists can influence leptin expression MDA-MB-231 and MCF-7, breast cancer cells were treated for 24 h with 0.5 μM of CGZ and GW in the presence or absence of the antagonist T0. Q-RT-PCR showed that submolar concentration of agonists stimulates the expression of leptin mRNA compared to control (DMSO). In detail, after treatment with 0.5 μM of CGZ, leptin mRNA increases of 2-fold in MDA-MB-231 and of 1.2-fold in MCF-7. Similar increment was observed with GW (1.6-fold in MDA-MB-231 and 1.4-fold in MCF-7; Fig. 2a).

Next experiments using a specific leptin ELISA assay demonstrated 0.5 μM CGZ or 0.5 μM GW stimulates the secretion of leptin to conditioned medium in MDA-MB-231 (1.4-fold with CGZ and 1.2-fold with GW vs. DMSO); in MCF-7, CGZ does not induce any change while GW induces an increment of 1.2-fold versus DMSO (Fig. 2b). In addition, the

TABLE 1. Effects of PPARγ ligands (CGZ and GW) on breast cancer cell viability*

	MDA-MB-231			MCF-7		
	0.5 μM	5 μM	10 μM	0.5 μM	5 μM	10 μM
CGZ	2.3 ± 0.052	1.7 ± 0.044	1 ± 0.054	3.1 ± 0.048	2.5 ± 0.053	1.2 ± 0.05
CGZ + T0	1.6 ± 0.051	0.9 ± 0.057	0.8 ± 0.049	1.7 ± 0.044	1.3 ± 0.056	1.2 ± 0.047
GW	2.5 ± 0.050	2.4 ± 0.048	1.3 ± 0.046	1.5 ± 0.042	2.7 ± 0.052	1.5 ± 0.046
GW + T0	1.1 ± 0.045	1 ± 0.055	0.8 ± 0.045	1.2 ± 0.052	1.2 ± 0.054	1.1 ± 0.043

Data, representative of three different experiments each performed in triplicate, are presented as fold change ± of cell viability relative to the control, $P \leq 0.05$.
*DMSO values are taken as = 1, and treatment values represent fold increase relative to DMSO.

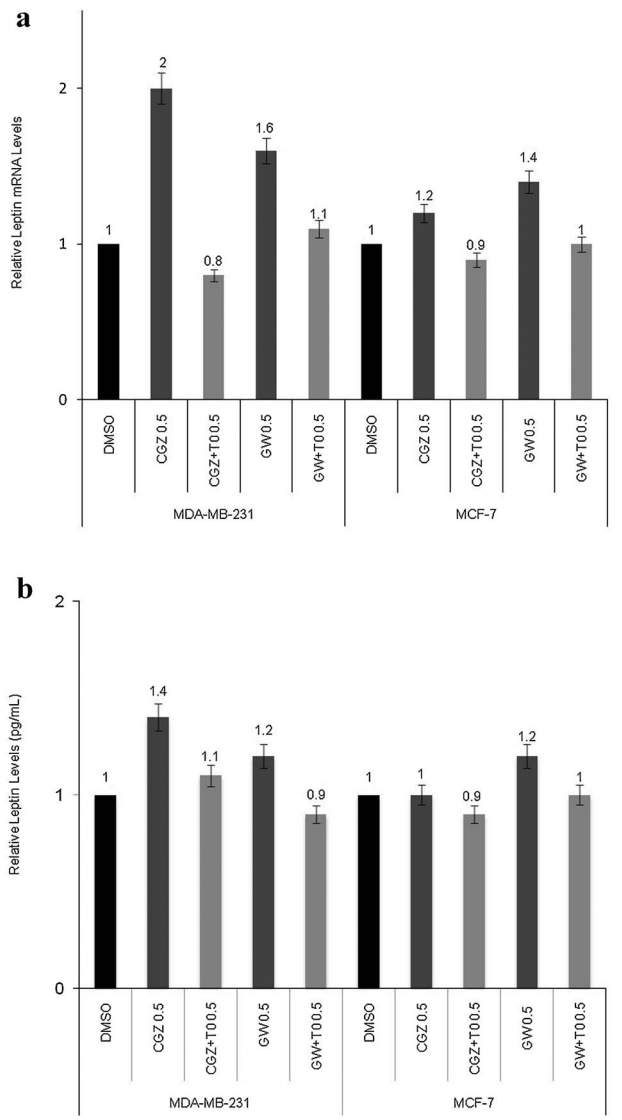

Fig. 2. Effects of submolar doses of CGZ and GW on leptin production. a: The abundance of leptin mRNA was studied using QRT-PCR in MDA-MB-231 and MCF-7 cells treated with 0.5 μM of CGZ/GW, alone or in combination with T0, for 24 h, relative to cells treated with DMSO as described in Materials and Methods Section. The graphs represent the increase of leptin mRNA relative to the increase of constitutive mRNA control (β-actin) in the same sample ± SD. b: With ELISA assay, the abundance of secreted leptin (pg/ml) was analyzed in conditioned medium of cells treated with 0.5 μM of CGZ/GW, alone or in combination with T0, for 24 h, relative to cells treated with DMSO as described in Materials and Methods Section. The differences between the three conditions (CGZ/GW/T0) and DMSO were statistically significant ($P < 0.05$).

combined treatment with the antagonist blocked the effects of the agonists both for mRNA and proteins levels (Fig. 2a,b).

Submolar concentrations of CGZ increase the association of PPARγ and reduce the binding of Sp1 with the proximal leptin promoter

Sp1 is a transcription factor able to interact with specific motifs within the leptin gene promoter under certain stimuli (Bartella et al., 2008).

Using ChIP assays we investigated the effects of CGZ on the binding of PPARγ and Sp1 to the proximal leptin promoter region (from −488 to +26). Contrary to earlier findings in rat adipocytes (Hollenberg et al., 1997), we found that in MDA-MB-231 and MCF-7 breast cancer cells, PPARγ directly binds this region. Specifically, the treatment with 0.5 μM CGZ for 24 h increased PPARγ loading on this DNA region by 2.1-fold in MDA-MB-231 and by 2.5-fold in MCF-7 cells. In parallel, CGZ treatment reduced Sp1 binding to this DNA region by 0.7-fold in MDA-MB-231 and by 0.3-fold in MCF-7 cells (Fig. 3a). Chromatin samples precipitated with control antibodies did not reveal any Q-RT-PCR products (data not shown).

The differential association of PPARγ and Sp1 with the proximal leptin promoter was not related to differences in the nuclear abundance of these factors in response to CGZ treatment. In fact, we only noticed a 20% reduction of PPARγ and a 10% increment of Sp1 nuclear levels after 24 h of CGZ treatment in MDA-MB-231 cells. In MCF-7 cells, the levels of both factors remained unchanged (Fig. 3b).

Effects of submolar concentrations of PPARγ agonists on VEGF expression

Since leptin is able to increase VEGF expression (Gonzalez-Perez et al., 2010) and PPARγ might regulate VEGF transcription (Biscetti et al., 2008), we assessed VEGF mRNA levels following the treatment with 0.5 μM of CGZ and GW in MCF-7 and MDA-MB-231 cells (Fig. 4a). We found that the agonists activated VEGF expression in MDA-MB-231 cells by 1.5-fold with CGZ and 1.2-fold with GW, but not in MCF-7 cells (Fig. 4a).

Interestingly, using specific ELISA assays, we found that 0.5 μM of CGZ or GW stimulated the secretion of VEGF in both cell lines (Fig. 4b). Specifically, VEGF secretion increased 1.8-fold with CGZ and 1.5-fold with GW in MDA-MB-231, while in MCF-7 a 1.2- and 1.6-fold increase was noted with CGZ and GW versus DMSO, respectively (Fig. 4b). In addition, the combined treatment with the antagonist blocked the effects of the agonists both for mRNA and proteins levels (Fig. 4a,b).

Effects of submolar concentrations of CGZ on the invasion of MDA-MB-231 and MCF-7 breast cancer cells

Leptin and VEGF are known motogenic factors for breast cancer cells (Bernatchez et al., 2002; Surmacz, 2007). Since both these proteins were induced by PPARγ agonists, we assayed whether submolar concentration of CGZ can induce invasive capacities of MDA-MB-231 and MCF-7 breast cancer cells (Fig. 5a). We found that relative to controls, the treatment with

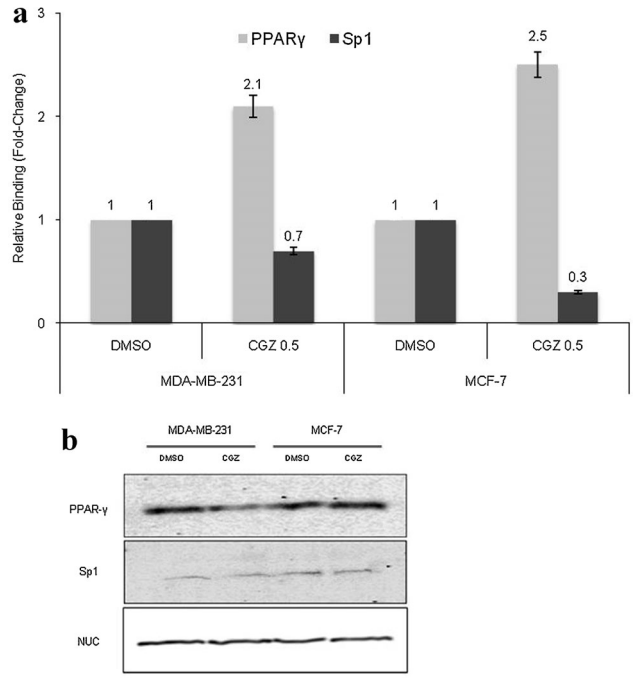

Fig. 3. Submolar CGZ increases the association of PPARγ and reduces the binding of Sp1 with the proximal leptin promoter. a: The binding of PPARγ and Sp1 to the proximal leptin promoter region (−488/+26) was tested by ChIP and QRT-PCR in MDA-MB-231 and MCF-7 cells treated with 0.5 μM of CGZ or DMSO for 24 h, as described in Materials and Methods Section. b: The expression of nuclear Sp1 and PPARγ was analyzed by WB in 100 μg of nuclear proteins obtained from cells treated with 0.5 μM of CGZ or DMSO for 24 h, as described in Materials and Methods Section. Protein loading was controlled by re-probing the WB filter for the expression of a nuclear marker nucleolin (NUC).

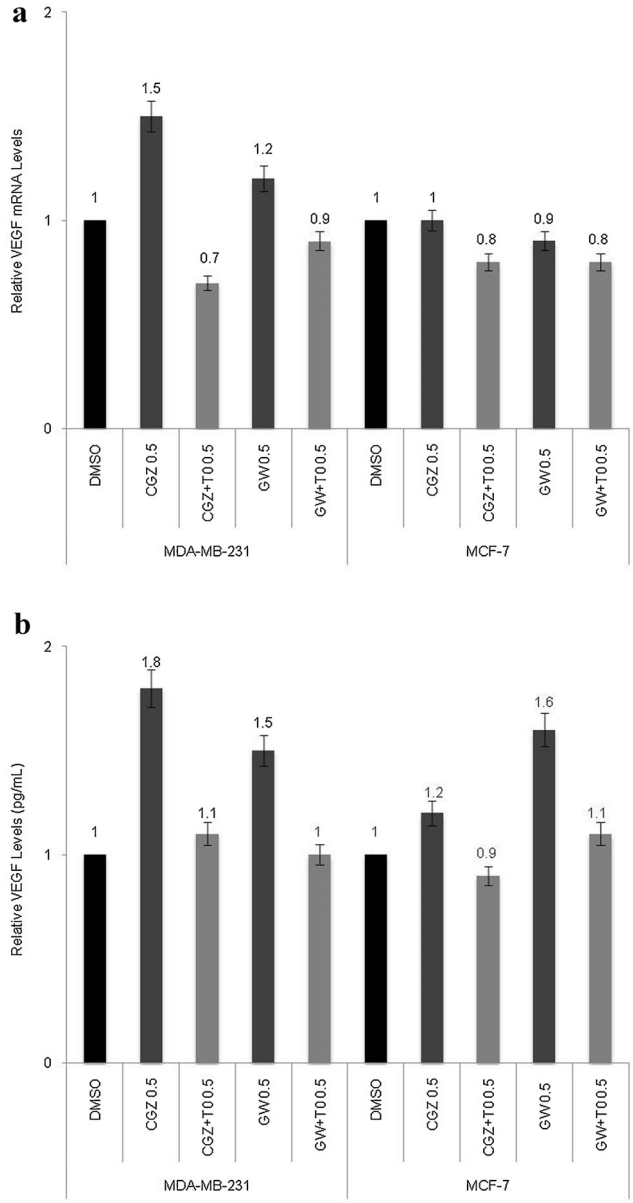

Fig. 4. Effects of submolar doses of CGZ and GW on VEGF production. a: The abundance of VEGF mRNA was studied using QRT-PCR in MCF-7 and MDA-MB-231 cells treated with 0.5 μM of CGZ/GW, alone or in combination with T0, for 24 h, relative to cells treated with DMSO as described in Materials and Methods Section. The graphs represent the increase of VEGF mRNA relative to the increase of constitutive mRNA control (β-actin) in the same sample ± SD. b: With ELISA assay, the abundance of secreted VEGF (pg/ml) was analyzed in conditioned medium of cells treated with 0.5 μM of CGZ/GW, alone or in combination with T0, for 24 h, relative to cells treated with DMSO as described in Materials and Methods Section. The differences between the two conditions (CGZ/GW/TO and DMSO) were statistically significant ($P < 0.05$).

0.5 μM CGZ for 24 h increased the number of invading MDA-MB-231 and MCF-7 cells by 1.9- and 3.2-fold respectively (Fig. 5b).

Discussion

The role of PPARγ agonists in cancer is unclear. The evidence from preclinical and clinical studies suggests that some PPARy agonists might promote cancer development. For instance, Piccinni et al. (2011) reported an association between the use of pioglitazone and the onset of bladder cancer, while Lewis et al. (2011) noted that only long-term (over 2 years), but not short-term, use of pioglitazone was weakly associated with increased risk for this disease. In addition, several studies employing in vitro and in vivo models indicated that PPARy agonists might be involved in breast and colon cancer (Clay et al., 2001; Fujita et al., 2003; Saez et al., 2004; Choi et al., 2008; Talbert et al., 2008).

Here, we studied the effects of CGZ and GW on the expression of two oncogenic proteins, leptin and VEGF in cellular models of breast cancer. We found that submolar concentrations of CGZ and GW increased leptin mRNA and protein levels in both studied cell lines (Fig. 2). These results are in contrast to what was observed in rat adipocytes where thiazolidinediones at doses ranged between 5 and 500 nM downregulated leptin gene expression (Kallen and Lazar, 1996). These differences could be attributed to different cell context or different PPARγ-specific ligands (CGZ and GW vs. BRL49653). In humans, PPARγ agonists (e.g., thiazolidinediones pioglitazone and rosiglitazone) are administered at low-medium mg/day doses, which correspond to sub- to med-M concentrations in vitro. While, some authors demonstrated that such doses had no effect on breast cancer cell proliferation and circulating fasting plasma leptin concentrations (Nolan et al., 1996; Yee et al., 2007), their effects on intraorgan leptin levels were never assessed and remain unknown.

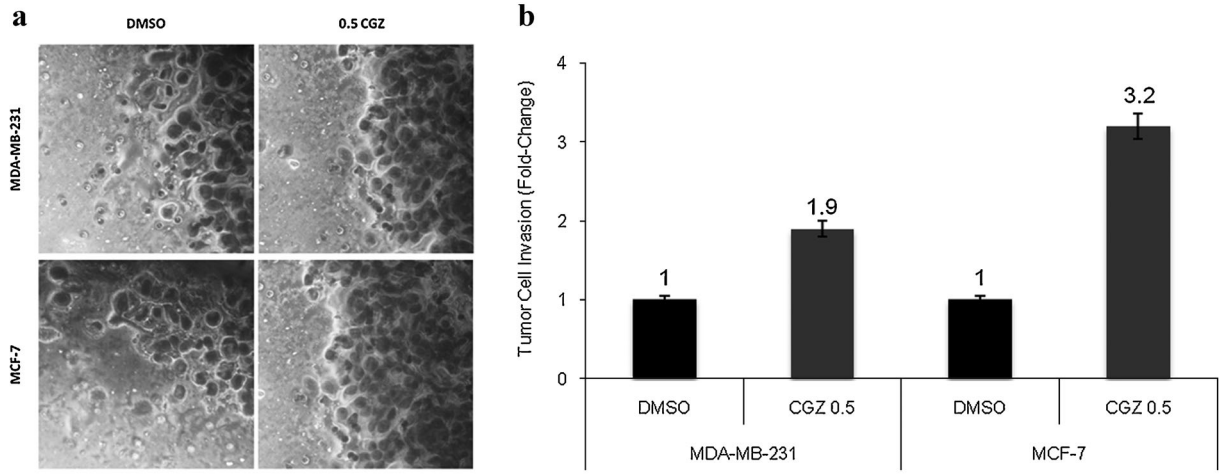

Fig. 5. Effects of submolar concentrations of CGZ on the invasion of MDA-MB-231 and MCF-7 breast cancer cells. Matrigel assay of MDA-MB-231 and MCF-7 breast cancer cells after treatment with 0.5 μM CGZ for 24 h compare to DMSO. a: Cell invasion assay was performed using BioCoat invasion chambers as described in Materials and Methods Section. Migrated cells were photographed under a microscope (magnification, 20×). b: The invasiveness was calculated as the percentage invasion through the matrigel relative to the migration through the control membrane. Data represent the mean ± SD of three independent experiments.

In our study, the CGZ-dependent upregulation of leptin mRNA and protein levels coincided with increased association of PPARγ to the proximal leptin promoter (−488 to +26) and reduced binding of Sp1 to this DNA region (Fig. 3a). The differential association of PPARγ and Sp1 with the proximal leptin promoter was not related to differences in the nuclear abundance of these factors in response to CGZ treatment (Fig. 3b). We speculate that PPARγ and Sp1 binding sites in the leptin promoter are overlapping, and the treatment with TZD could result in displacement of basal Sp1 levels. Our observations are at variance with the results obtained in primary rat adipocytes, where the leptin promoter region at −65/+9 was negatively regulated by PPARγ ligands via a mechanism not involving direct PPARγ binding to DNA (Hollenberg et al., 1997).

We also demonstrated that 0.5 μM of agonists increased VEGF mRNA only in MDA-MB-231. In MCF-7, PPARγ agonists did not alter VEGF mRNA levels but modulated protein levels through yet unclear mechanisms (Fig. 4).

We speculate that higher levels of leptin and VEGF can, at least in part, explain observed increased in viability (Fig. 1) and migration (Fig. 5) of MDA-MB-231 and MCF-7 cells under 0.5 μM of CGZ treatment.

In general, our results are consistent with the finding that PPARγ agonists can stimulate angiogenesis through upregulation of VEGF and other yet unidentified pro-angiogenic factors (Biscetti et al., 2008) and leptin could be one of these factors, stimulating angiogenesis independently (Anagnostoulis et al., 2008) or through enhanced expression of VEGF (Cao et al., 2001).

In summary, our study, for the first time, provided evidence that submolar concentrations of PPARγ agonists may induce survival and migration of MDA-MB-231 and MCF-7 breast cancer cells probably through up-regulation of leptin and VEGF, both factors implicated in tumor growth and progression.

Together with previous reports on proliferative and angiogenic effects of PPARγ agonists observed with low doses of these compounds (Clay et al., 2001) and considering recent data emerging from clinical studies (Lewis et al., 2011; Piccinni et al., 2011; Colmers et al., 2012; Kermode-Scott, 2012; Mamtani et al., 2012) our results add to the debate about relative risk-benefit profile of PPARγ targeting drugs.

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