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Peroxisome proliferator-activated receptor gamma activates fas ligand gene promoter inducing apoptosis in human breast cancer cells

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Abstract In just over a decade, apart from established metabolic actions, peroxisome proliferator-activated receptor gamma (PPAR γ) has evolved as key therapeutic target in cancer disease. Fas ligand (FasL), a trans-membrane protein, induces apoptosis by crosslinking with the Fas receptor. Despite the FasL relevance, little is available on the regulation of its expression. In the current study, we explored for the first time the potential role of PPAR γ in triggering apoptotic events through the Fas/FasL pathway in breast cancer cells. In MCF7 cells, by reverse transcription-polymerase chain reaction and Western blotting, we showed that the synthetic PPAR γ ligand rosiglitazone (BRL) enhanced FasL expression, that was abrogated by a specific PPAR γ antagonist GW9662. Transient transfection assays demonstrated that BRL transactivated human FasL promoter gene in a PPAR γ -dependent manner. Progressive 5' deletion analysis has identified a minimal promoter

fragment spanning nucleotides from –318 to –237 bp, which is still sensitive to BRL treatment. FasL promoter activity was abrogated by mithramycin, suggesting an involvement of Sp1 transcription factor in PPAR γ action. Electrophoretic mobility shift and chromatin immunoprecipitation assays demonstrated that BRL increased the binding of PPAR γ and Sp1 to the Sp1 sequence located within the FasL gene promoter. The role of PPAR γ and Fas/FasL pathways in BRL-induced apoptotic events was assessed by caspase 8 cleavage in the presence of GW as well as PPAR γ and FasL RNA interferences. Our results indicate that PPAR γ positively regulates the FasL gene expression also in MDA-MB231 and in BT20, revealing a new molecular mechanism by which BRL induces apoptosis in breast cancer cells.

Keywords PPAR γ · FasL · Apoptosis · Caspase 8 · Breast cancer

Abbreviations

PPAR γ	Peroxisome proliferator-activated receptor gamma
BRL	Rosiglitazone
MDA	MDA-MB231
RT-PCR	Reverse transcription-polymerase chain reaction
WB	Western blotting
FasL	Fas ligand
EMSA	Electrophoretic mobility shift assay
ChIP	Chromatin immuno-precipitation

Introduction

Since its discovery at previous decade, it became clear that peroxisome proliferator-activated receptor gamma (PPAR γ)

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is an important modulator in the regulation of complex pathways of mammalian cells' metabolism and recently implicated in cancer [1–3]. Several antineoplastic effects, such as induction of apoptosis and differentiation, have been described as a result of PPAR γ -mediated action both in vitro and in vivo [4–10], although the molecular mechanisms by which PPAR γ causes these effects remain to be fully elucidated. PPAR γ is able to be activated by ligands of physiological or pharmacological origin [11]. Synthetic PPAR γ ligands are the thiazolidinediones (TZDs), a class of oral antidiabetic agents, which exert anti-tumoral effects depending on the experimental and cellular settings and in a PPAR γ -dependent or PPAR γ -independent fashion [12, 13].

In mammals, apoptosis can be initiated by two major routes: the intrinsic and extrinsic death pathways [14]. The intrinsic pathway is triggered in response to a variety of apoptotic stimuli that produce damage within the cell, including anticancer agents, oxidative damage, UV irradiation, and is mediated through the mitochondria. The extrinsic pathway is activated by extracellular ligands able to induce oligomerization of death receptors, such as Fas (also called CD95 or APO1) or other members of the tumor necrosis factor receptor superfamily leading to apoptosis [15].

Fas ligand (FasL), a type II transmembrane protein expressed on the surface of cells, induces apoptotic cell death by binding to its receptor Fas [16, 17]. This binding results in recruitment of the Fas-associated death domain (FADD) protein and caspase 8 zymogens to the receptor and the formation of the death-inducing signalling complex, after which the caspases cascade can be activated [18]. Defects in the Fas/FasL apoptotic signalling pathway provide a survival advantage to cancer cells and may be implicated in tumorigenesis [19]. Recent evidence has indicated the Fas pathway in the induction of tumor cell death and FasL expression has been observed in different tumors including breast cancer [20–22]. It has also been suggested that FasL functions as an autocrine/paracrine mediator of apoptosis induced by DNA-damaging anticancer chemotherapeutic agents [23, 24].

In the past few years, we have investigated different molecular mechanisms through which PPAR γ induces anti-proliferative effects, cell cycle arrest and apoptosis in human MCF7 breast cancer cells [25, 26]. In this study, we reported for the first time that PPAR γ directly activates FasL promoter gene inducing apoptotic events in breast cancer cells.

Materials and methods

Cell cultures

Human MCF7 (a gift from Dr. Ewa Surmacz, Sbarro Institute for Cancer Research and Molecular Medicine,

Philadelphia, USA) MDA-MB231 (ER α -negative, MDA) breast cancer cells were grown in DMEM plus glutamax containing 10% fetal calf serum (FCS) (Invitrogen, Milan, Italy) and 1 mg/ml penicillin–streptomycin (P/S). BT20 (insulin receptor substrate-1, IRS-1 negative) breast cancer cells were grown in MEM added as DMEM.

Reagents

Rosiglitazone (BRL49653, BRL) was from Alexis (San Diego, CA USA), the irreversible PPAR γ -antagonist GW and the Sp1 specific inhibitor, mithramycin were from Sigma (Milan, Italy).

Plasmids

The firefly luciferase reporter plasmids containing FasL promoter wild-type (FasL Luc-0) or its deletion mutants (FasL Luc-3 and FasL Luc-4) were kind gifts from Dr. Carlos V. Paya (Mayo Clinic, Rochester, Minnesota, USA). Deletion of Sp1 sequence in FasL gene promoter was generated by PCR using as template FasL Luc-3 (p-318 construct). The resulting plasmid encoding the human FasL gene promoter containing the desired deletion was designed FasL Luc-3 Δ Sp1 and the sequence was confirmed by nucleotide sequence analysis [27]. As an internal transfection control, we co-transfected the plasmid pRL-CMV (Promega Corp., Milan, Italy) that expresses Renilla luciferase enzymatically distinguishable from firefly luciferase by the strong cytomegalovirus enhancer/promoter.

Immunoblotting

Cells were grown in 10 cm dishes to 70–80% confluence and exposed to treatments in serum free medium (SFM) as indicated. Cells were then harvested in cold phosphate-buffered saline (PBS) and resuspended in lysis buffer containing 20 mM HEPES pH 8, 0.1 mM EDTA, 5 mM MgCl₂, 0.5 M NaCl, 20% glycerol, 1% NP-40, inhibitors (0.1 mM Na₃VO₄, 1% PMSF, 20 mg/ml aprotinin). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA USA). A 60 μ g portion of protein lysates was used for WB resolved on a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with antibodies (Abs) directed against the FAS, FasL and PPAR γ (Santa Cruz Biotechnology, CA USA), caspase 8 (Biomol International, Butler Pike Plymouth, PA USA). As internal control, all membranes were subsequently stripped (glycine 0.2 M, pH 2.6 for 30 min at room temperature) of the first antibody and reprobed with anti- β -actin Ab (Santa Cruz Biotechnology).

The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with

the appropriated secondary antibodies peroxidase-coupled and revealed using the enhanced chemiluminescence system (ECL system, Amersham Pharmacia, Buckinghamshire, UK). Blots were then exposed to film (Kodak film, Sigma). The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

Reverse transcription-polymerase chain reaction (RT-PCR) assay

MCF7 cells were grown in 10 cm dishes to 70–80% confluence and exposed to treatments for 24 h in SFM. Total cellular RNA was extracted using TRIZOL reagent (Invitrogen) as suggested by the manufacturer. The purity and integrity were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. The evaluation of gene expression was performed by semiquantitative RT-PCR method as previously described [28]. For FasL and the internal control gene 36B4, the primers were: 5'-GGA ATG GGA AGA CAC CTA TGG A-3' (FasL forward) and 5'-AGA GAG AGC TCA GAT ACG TTG AC-3' (FasL reverse), 5'-CTC AAC ATC TCC CCC TTC TC-3' (36B4 forward) and 5'-CAA ATC CCA TAT CCT CGT CC-3' (36B4 reverse) to yield respectively products of 299 bp with 25 cycles and 408 bp with 12 cycles. The results obtained as optical density arbitrary values were transformed to percentage of the control (percent control) taking the samples from untreated cells as 100%.

Transient transfection assay

MCF7 cells were transferred into 24 well plates with 500 μ l of regular growth medium/well the day before transfection. The medium was replaced with SFM on the day of transfection, which was performed using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany) with a mixture containing 0.5 μ g of promoter-luciferase reporter plasmid and 5 ng of pRL-CMV. After 24 h transfection, treatments were added in SFM as indicated and cells were incubated for further 24 h, cells were pre-treated for 2 h with mithramycin or GW where necessary. Firefly and Renilla luciferase activities were measured using the Dual Luciferase Kit (Promega). The firefly luciferase values of each sample were normalized by Renilla luciferase activity and data were reported as Relative Light Units (RLU) values.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from MCF7 cells were prepared as previously described [29]. Briefly, MCF7 cells plated into 10 cm

dishes were grown to 70–80% confluence shifted to SFM for 24 h and then treated with 1 μ M BRL for 6 h. Thereafter, cells were scraped into 1.5 ml of cold PBS. Cells were pelleted for 10 s and resuspended in 400 μ l cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were centrifuged for 10 s and the supernatant fraction discarded. The pellet was resuspended in 50 μ l of cold Buffer B (20 mM HEPES-OH pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated in ice for 20 min for high-salt extraction. Cellular debris were removed by centrifugation for 2 min at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at -70°C. The probe was generated by annealing single stranded oligonucleotides and labeled with [γ -³²P] ATP (Amersham Pharmacia) and T4 polynucleotide kinase (Promega) and then purified using Sephadex G50 spin columns (Amersham Pharmacia). The DNA sequence, containing the Sp1, NFAT and NF κ B sites, obtained from the native FasL promoter gene used as probe or as cold competitor is the following: 5'-A AAT TGT GGG CGG AAA CTT CCA GGG-3' (Sigma). As cold competitor we also used Δ Sp1 oligonucleotide: 5'-AT TGT GTT CGG AAA CTT CCA GGG-3', Δ NFAT oligonucleotide: 5'-A AAT TGT GGG CGG TCA CT TCCA GGG-3' and Δ NF κ B oligonucleotide: 5'-A AAT TGT GGG CGG AAA CAT ATA GGG-3' (Sigma). The protein binding reactions were carried out in 20 μ l of buffer [20 mM Hepes pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glycerol, 1 mg/ml BSA, 50 μ g/ml poly dI/dC] with 50,000 cpm of labeled probe, 5 μ g of MCF7 nuclear protein and 5 μ g of poly (dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitors oligonucleotides. For in vitro mithramycin treatment, 100 nM mithramycin were incubated with the labelled probe for 30 min at 4°C before the addition of nuclear extract. For the experiments involving the anti-PPAR γ and anti-Sp1 Abs (Santa Cruz Biotechnology), the reaction mixture was incubated with Ab at 4°C for 30 min before addition of labeled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 \times Tris borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at -70°C.

Chromatin immunoprecipitation (ChIP) and Re-ChIP assays

For ChIP assay, MCF7 cells were grown in 10 cm dishes to 50–60% confluence, shifted to SFM for 24 h and then treated with 1 μ M BRL for 1 h or pre-incubated with

mithramycin for 1 h where required. Thereafter, cells were washed twice with PBS and crosslinked with 1% formaldehyde at 37°C for 10 min. Next, cells were washed twice with PBS at 4°C, collected and resuspended in 200 µl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1) and left on ice for 10 min. Then, cells were sonicated four times for 10 s at 30% of maximal power (Sonics, Vibra Cell 500 W) and collected by centrifugation at 4°C for 10 min at 14,000 rpm. The supernatants were diluted in 1.3 ml of IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 16.7 mM NaCl) followed by immunoclearing with 80 µl of sonicated salmon sperm DNA/protein A agarose (UBI, DBA Srl, Milan—Italy) for 1 h at 4°C. The precleared chromatin was immunoprecipitated with anti-PPAR γ Ab and re-immunoprecipitated with anti-RNA Pol II Ab (Santa Cruz Biotechnology). At this point, 60 µl salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 h at 4°C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl), Wash B (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl), and Wash C (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8.1), and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immunocomplexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃). The eluates were reverse crosslinked by heating at 65°C and digested with proteinase K (0.5 mg/ml) at 45°C for 1 h. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. 2 µl of 10 mg/ml yeast tRNA (Sigma) were added to each sample and DNA was precipitated with 70% ethanol for 24 h at –20°C, and then washed with 95% ethanol and resuspended in 20 µl of TE buffer. A 5 µl volume of each sample was used for PCR amplification with the following primers flanking a sequence of FasL promoter: 5'-TAC CCC CAT GCT GAC CTG CTC-3' (forward) and 5'-ACG GGA CCC TGT TGC TGA CTG-3' (reverse) corresponding to the –396 to –76 region (Gene Bank, accession number: AF027385). The PCR conditions were 45 s at 94°C, 40 s at 58°C, and 90 s at 72°C. The amplification products obtained in 30 cycles were analysed in a 2% agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without DNA sample. The specificity of reactions was ensured using normal mouse and rabbit IgG (Santa Cruz Biotechnology).

ChIP/immunoblot assay

For ChIP/Immunoblot assay, MCF7 cells were subjected to the procedure of ChIP assay, as above described, until we obtained the immunocomplexes. At this time, we added the

Laemmli buffer to the immunocomplexes and performed the WB analysis, as described by the Upstate protocol. The specificity of reactions was ensured using normal rabbit IgG (Santa Cruz Biotechnology).

RNA interference (RNAi)

Cells were plated in 6 well dishes with regular growth medium the day before transfection to 60–70% confluence. On the second day the medium was changed with SFM without P/S and cells were transfected with 25 bp RNA duplex of stealth RNAi targeted human FasL mRNA sequence 5'-GCC CAU UUA ACA GGC AAG UCC AAC U-3' (Invitrogen), with 25 bp RNA duplex of validate RNAi targeted human PPAR γ mRNA sequence 5'-GCC UGC AUC UCC ACC UUA UUA UUC U-3' or with a stealth RNAi control (Invitrogen) to a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 5 h the transfection medium was changed with SFM in order to avoid Lipofectamine 2000 toxicity and cells were exposed to 1 µM BRL for the next 48 h and then lysed as described for WB analysis or treated for 72 h and then collected as described for the DNA fragmentation.

DNA fragmentation

DNA fragmentation was determined by gel electrophoresis. Cells were grown in 10-cm dishes to 70% confluence, PPAR γ or control RNAis and then treated with 1 µM BRL and/or 10 µM GW. After 72 h cells were collected and washed with PBS and pelleted at 1,800 rpm for 5 min. The samples were resuspended in 0.5 ml of extraction buffer (50 mM, Tris–HCl, pH 8; 10 mM EDTA, 0.5% SDS) for 20 min in rotation at 4°C. DNA was extracted with phenol–chloroform three times and once with chloroform. The aqueous phase was used to precipitate acids nucleic with 0.1 volumes or of 3 M sodium acetate and 2.5 volumes cold EtOH overnight at –20°C. The DNA pellet was resuspended in 15 µl of H₂O treated with RNase A for 30 min at 37°C. The absorbance of the DNA solution at 260 and 280 nm was determined by spectrophotometry. The extracted DNA (40 µg/lane) was subjected to electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and then photographed.

Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

Results

MCF7 breast cancer cells express both Fas and FasL

Previous studies have shown that MCF7 cells coexpress both Fas and FasL, indeed controversial data are reported in this context [30–32]. To evaluate if Fas and FasL are expressed in our MCF7 cells, we did WB analysis of cellular lysates and found that they do indeed express both proteins (Fig. 1a).

BRL up-regulates FasL expression in MCF7 cells

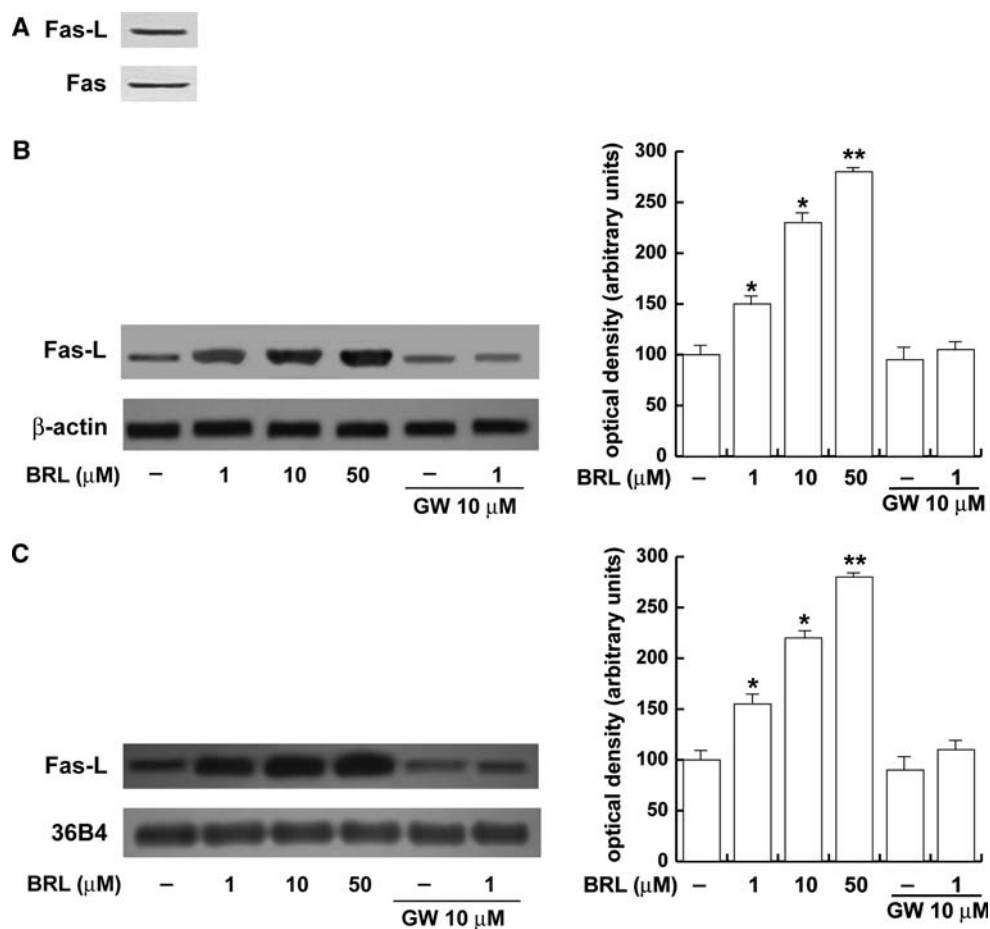
In order to understand whether PPAR γ triggers apoptotic events through Fas/FasL signalling, we first evaluated the ability of PPAR γ agonist BRL to modulate FasL expression in MCF7 cells. A BRL dose-dependent increase in FasL content was observed by WB after 24 h of treatment (Fig. 1b). Furthermore, by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) method, after 24 h upon increasing BRL concentration, we showed that BRL was able to upregulate FasL gene expression in a dose-dependent manner (Fig. 1c). The BRL action on FasL

expression was reversed when we used GW9662 (GW), a specific PPAR γ antagonist, (Fig. 1b and c) implying a PPAR γ -dependent action in MCF7 cells.

PPAR γ transactivates FasL promoter in MCF7 cells

Next, we evaluated whether one of the mechanisms involved in PPAR γ -mediated increase of FasL expression could be a direct modulation of the transcriptional activity of its promoter. Transient transfection assays were performed in MCF7 cells using the luciferase reporter constructs containing the region of the human FasL promoter gene spanning from –2365 to –2 bp relative to the translation initiation site (FasL Luc-0) or progressive 5' deletions of the FasL promoter, FasL Luc-3 (from –318 to –2 bp) and FasL Luc-4 (from –237 to –2 bp) (Fig. 2a). Of interest, differences in the basal transcriptional activity were observed among constructs. The results shown in Fig. 2b indicate that transcription from the FasL Luc-0 construct was detectable, but strong and increasing luciferase activity was evidenced with FasL Luc-3 and FasL Luc-4 constructs, suggesting the presence of potential

Fig. 1 BRL up-regulates FasL protein and mRNA expression in MCF7 cells. **(a)** Immunoblots of FasL and Fas from MCF7 breast cancer cells. **(b)** Immunoblots of FasL from MCF7 cells treated for 24 h with vehicle (–), increasing BRL concentrations, 10 μ M GW alone or in combination with 1 μ M BRL. β -actin was used as loading control. The *side panel* shows the quantitative representation of data (mean \pm S.D.) of three independent experiments including that of **b**. **(c)** Semiquantitative RT-PCR evaluation of FasL mRNA expression. MCF7 cells were treated as in **b**. 36B4 mRNA levels were determined as control. The *side panel* shows the quantitative representation of data (mean \pm S.D.) of three independent experiments including that of **c** after densitometry and correction for 36B4 expression. * P < 0.05 BRL-treated vs untreated cells ** P < 0.01 BRL-treated vs. untreated cells



negative regulatory regions located within –2365 and –318 bp, consistent with a previous study [33].

Using the FasL Luc-3 construct, the promoter activity increased upon BRL treatment in a dose-dependent manner, while this effect was abrogated by GW, indicating that FasL promoter activation depends on the presence of a functional PPAR γ (Fig. 2c). BRL failed to enhance the transcriptional activity when cells were transfected with FasL Luc-4 construct (Fig. 2c), suggesting that the segment located between –318 and –237 relative to the translation initiation site is crucial for BRL-responsiveness. In particular, this region contains three DNA motifs known to bind the transcription factors Sp1 (named according to the original purification scheme that included Sephacryl and phosphocellulose columns) [34], nuclear factor of activated T cells (NFAT) and nuclear factor- κ B (NF κ B) (Fig. 2a) [33]. It was reported that Sp1 consensus sequence is necessary for basal transcription from the FasL promoter, and that Sp1 alone is sufficient to drive transcription from the promoter in vivo [33]. We sought to investigate whether Sp1 alone was sufficient to drive FasL transcription or if it required the presence of additional ones. By using a FasL Luc-3 construct deleted in the Sp1 site (Fas Luc-3 Δ Sp1),

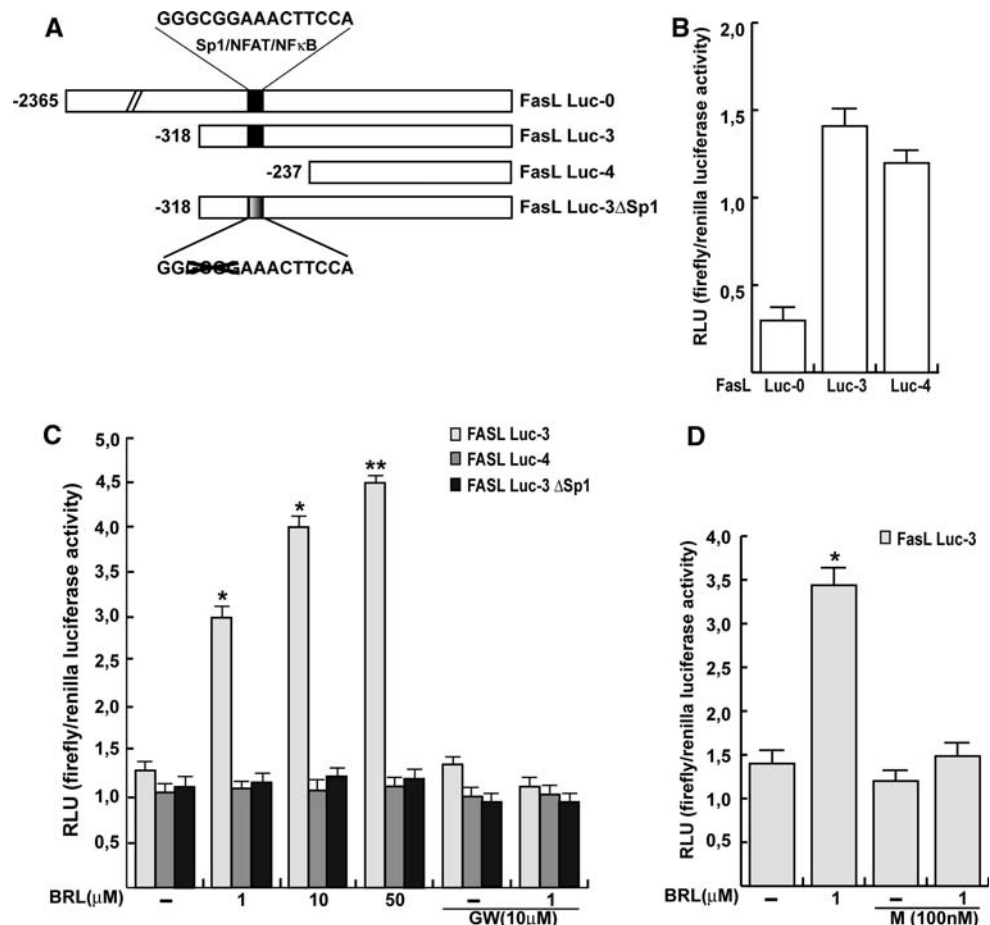
as shown in Fig. 2a, BRL was unable to transactivate FasL promoter (Fig. 2c). Mithramycin is a drug able to bind to GC boxes and then to inhibit Sp1 binding selectively blocking mRNA synthesis from genes that contain functional recognition sites both in vitro and in vivo [35]. Besides, as shown in Fig. 2d, in MCF7 cells transfected with FasL Luc-3 construct and treated with mithramycin, the FasL transactivation upon 1 μ M BRL treatment was prevented. Altogether these data strongly suggest that Sp1 plays an important role in regulating FasL transcription also in MCF7 cells.

PPAR γ interacts with FasL through Sp1 site in electrophoretic mobility shift assay (EMSA)

To gain further insight into the mechanism involved in the FasL transactivation induced by BRL, we performed EMSA using a synthetic oligodeoxyribonucleotide which contains the Sp1, NFAT and NF κ B DNA binding motifs and encompasses the DNA fragment of FasL promoter from –288 to –263 relative to the translation initiation site. We tested for possible interactions of PPAR γ with

Fig. 2 BRL transactivates FasL promoter gene in MCF7 cells.

(a) Schematic map of the FasL promoter fragments used in this study. (b) Basal transcriptional activity of the different FasL-luciferase report constructs (Luc-0, Luc-3 and Luc-4). (c) MCF7 cells were treated for 24 h with vehicle (–), increasing BRL concentrations, 10 μ M GW alone or in combination with 1 μ M BRL. (d) MCF7 cells were treated for 24 h with vehicle (–), 1 μ M BRL, 100 nM mithramycin (M) alone or in combination with 1 μ M BRL. The luciferase activities were normalized to the Renilla luciferase as internal transfection control and data were reported as (RLU values. Columns are mean \pm S.D. of three independent experiments performed in triplicate. * P < 0.05 BRL-treated vs untreated cells, ** P < 0.01 BRL-treated vs untreated cells. RLU, Relative Light Units



each of the indicated transcription factors located within the above mentioned FasL promoter region.

In nuclear extracts from MCF7 cells we observed the formation of three specific complexes (Fig. 3, lane 1) which were increased upon BRL treatment (lane 2). The binding was abrogated by 100 fold molar excess of unlabelled probe (lane 3), demonstrating the specificity of the DNA-binding complex. To confirm that FasL transcription induced by BRL in MCF7 cells is regulated by Sp1, nuclear extracts were incubated with three different unlabelled oligonucleotides bearing internal mutations as reported in Materials and Methods.

An excess of unlabeled oligonucleotide containing mutations in the Sp1 site did not compete with the DNA binding of the protein complex (lane 4). In addition,

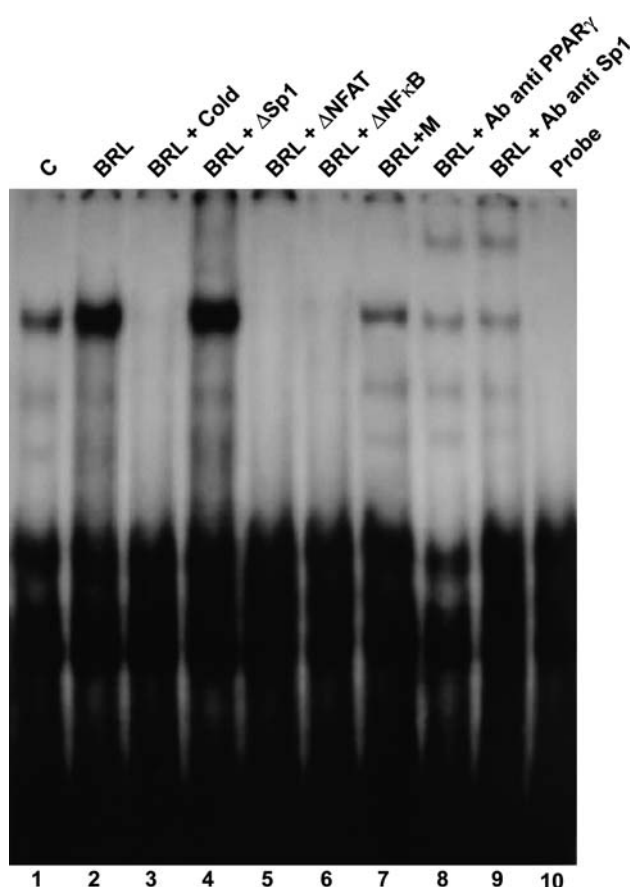


Fig. 3 PPAR γ binds to Sp1 site within FasL promoter region in EMSA. Nuclear extracts from MCF7 cells (lane 1) were incubated with a labeled sequence containing bases –288 to –263 of the wild-type FasL promoter and subjected to electrophoresis in a 6% polyacrylamide gel. In lane 2, nuclear extracts from MCF7 were treated with 1 μ M BRL. Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled FasL probe (lane 3) or 100 nM mithramycin (M) (lane 7). An excess of three unlabeled oligonucleotides Δ Sp1 (lane 4), Δ NFAT (lane 5) and Δ NF κ B (lane 6), was used. Anti-PPAR γ and anti-Sp1 Abs were incubated with nuclear extracts from MCF7 cells treated with 1 μ M BRL (lanes 8 and 9, respectively). Lane 10 contains probe alone

incubation with an excess of unlabeled oligonucleotides containing mutations of either NFAT or NF κ B competed both away all DNA-binding activity of the protein complexes bound to the 32 P-labeled wild type –288 to –263 FasL oligonucleotide (lane 5 and 6, respectively). Moreover, the addition of mithramycin in the reaction mixture strongly reduced the intensity of the bands (Fig. 3, lane 7). The involvement of PPAR γ and Sp1 in the DNA-binding complexes was confirmed by using the specific anti-PPAR γ (lane 8) and anti-Sp1 Abs (lane 9), since both induced supershift and immunodepletion of the bands.

Functional interaction of PPAR γ with FasL by chromatin immuno-precipitation (ChIP) assay

The interaction of PPAR γ with the FasL gene promoter was further investigated by ChIP assay. MCF7 chromatin was immunoprecipitated with the anti-PPAR γ Ab and then reprecipitated with the anti-RNA-Pol II or anti-Sp1 Abs. PCR was used to determine the recruitment of PPAR γ to the FasL region containing the Sp1 site. The results indicated that PPAR γ was weakly constitutively bound to the FasL promoter in untreated cells and this recruitment was increased upon BRL treatment, while mithramycin combined with BRL reversed this effect (Fig. 4a). In addition, by Re-ChIP assays an increased association of Sp1 was obtained and particularly the augmented RNA-Pol II recruitment indicated that a positive regulation of FasL transcription activity was induced by BRL (Fig. 4a). The physical interaction between PPAR γ and Sp1 proteins was strengthened by the ChIP/Immunoblot assay (Fig. 4b).

PPAR γ activates the Fas/FasL apoptotic pathway

Fas/FasL signalling when activated recruits adapter proteins and cysteine proteases such as caspase 8 leading to apoptosis [14]. To better define the action of PPAR γ on Fas/FasL pathway, we used GW as well as both PPAR γ and FasL RNA interferences (i) to evaluate the activation of caspase 8, key component of the extrinsic apoptotic process. By WB analysis our data showed that untreated MCF7 cells expressed the pro-form of caspase 8, while only after BRL exposure caspase 8 was activated as evidenced by the presence of its 11 kDa cleavage product (Fig. 5c). The active caspase 8 cleavage was absent in cells treated with GW alone or combined with BRL, or inhibiting both the expression of PPAR γ and FasL by the respective RNAis (Fig. 5c). As shown in Fig. 5a and b, PPAR γ and FasL RNAis were able to inhibit the two proteins expression respectively. These data demonstrated for the first time that PPAR γ triggers the apoptotic events via Fas/FasL signalling, showing that the extrinsic death

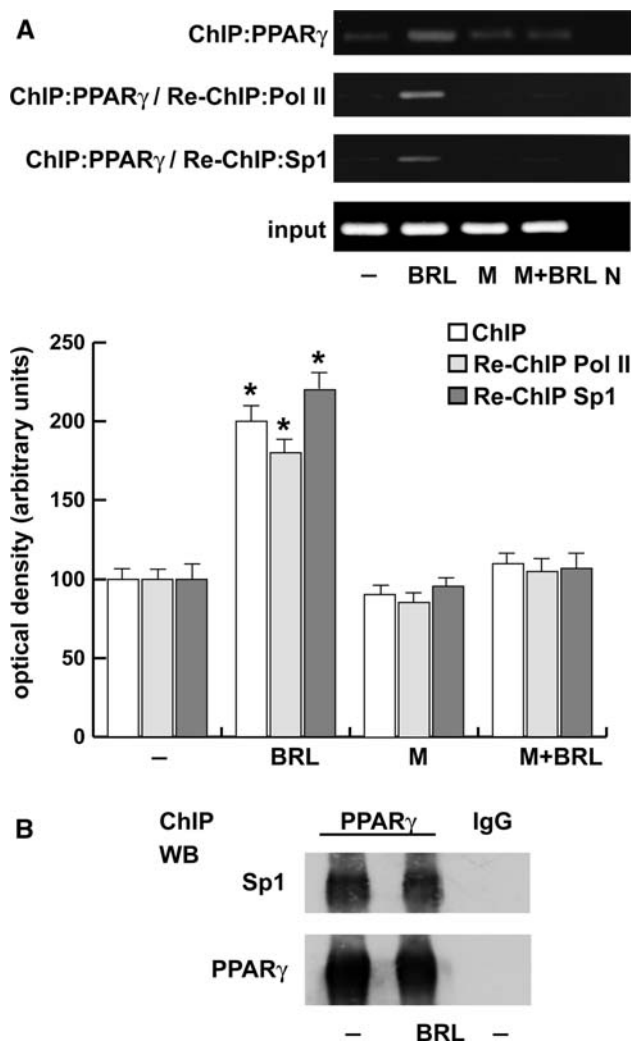


Fig. 4 Functional interaction between PPAR γ and FasL promoter. **(a)** MCF7 cells were treated as indicated, then cross-linked with formaldehyde and lysed. The soluble chromatin was immunoprecipitated with the anti-PPAR γ Ab, (Re-ChIP with the anti-RNA Pol II and anti-Sp1 Abs). The FasL promoter sequence containing Sp1 was detected by PCR with specific primers, as described in Materials and Methods. To control input DNA, FasL promoter was amplified from 30 μ l of initial preparations of soluble chromatin (before immunoprecipitations). In the bottom of the panel quantitative representation of data of three independent experiments (mean \pm S.D.) after densitometry. * P < 0.05 BRL-treated vs. untreated cells. M: mithramycin. N, negative control provided by PCR amplification without DNA sample. **(b)** Immunoblots of Sp1 and PPAR γ from MCF7 cells treated with BRL 1 μ M, in which chromatin was immunoprecipitated with the anti-PPAR γ antibody and IgG

pathway is a significant contributor in BRL-induced apoptosis in MCF7 cells.

The apoptotic process is associated with morphological changes and biochemical events such as nuclear condensation and fragmentation, the fragments correspond to strands of DNA that were cleaved at internucleosomal regions and create a 'ladder pattern' when electrophoresed on an agarose gel [36]. Because of its near universality,

internucleosomal DNA degradation is considered a diagnostic hallmark of cells undergoing apoptosis. Therefore, we studied DNA fragmentation assay under BRL treatment in MCF7 cells evidencing that the induced apoptosis is PPAR γ -dependent as it was reversed by the PPAR γ RNAi (Fig. 5d). These results indicate a positive crosstalk between PPAR γ and FasL that is responsible, at least in part, for BRL-induced apoptosis in MCF7 cells.

FasL is a common mechanism by which PPAR γ mediates apoptosis in breast cancer cells

Finally, we examined other human breast cancer cell lines to determine whether the involvement of FasL is unique to MCF-7 cells or it is a common mechanism by which PPAR γ mediates apoptosis in breast cancer. MDA and BT20 cells, both expressing Fas and FasL (Fig. 6a), showed a FasL upregulation upon BRL treatment, which was reduced by PPAR γ RNAi (Fig. 6b). DNA fragmentation assay under BRL treatment in MDA and BT20 cells confirmed that the PPAR γ -induced apoptosis involves Fas/FasL signalling (Fig. 6c), addressing a general mechanism in breast cancer cells.

Discussion

PPAR γ ligands are shown to be generally antiproliferative and to induce apoptosis in several malignant cell lineages [4, 6, 7, 9]. Additionally, PPAR γ is proved to be a regulator of the expression of many genes relevant to inhibit carcinogenesis [37]. Our previous studies evidenced that PPAR γ , through different pathways, is involved in BRL-induced growth arrest and apoptosis in MCF7 breast cancer cells [25, 26]. In the last report, where we explored the involvement of the p53 pathway, it appears that the PPAR γ -mediated apoptosis is not completely blocked by using the p53 antisense, suggesting that final PPAR γ action in this concern is complex and it may require a multifactorial coordination of different signalling cascades. In this finding, we investigated the potential of PPAR γ in inducing apoptotic events through a direct involvement of the Fas/FasL extrinsic apoptotic pathway in breast cancer cells.

The Fas/FasL signalling system plays an important role in chemotherapy-induced apoptosis in several different cell types [23, 24]. FasL is a member of the TNF superfamily that induces apoptosis in susceptible cells upon cross-linking of its own receptor, Fas [15, 16, 38, 39]. FasL is expressed on the cell membrane surface of activated T lymphocytes and cancer cells [40–42]. Significantly, constitutive down-regulation of Fas is involved in drug resistance [43] and associated with a poor prognosis in breast cancer [32].

Fig. 5 BRL induces the extrinsic apoptotic pathway in MCF7 cells. PPAR γ (a) and FasL (b) protein expression (evaluated by WB) in MCF7 cells transfected with a 25-nucleotide of RNA interference (RNAi) targeted human PPAR γ or FasL mRNA sequence respectively, or with control RNAi as reported in Materials and Methods or not transfected and treated for 48 h as indicated. β -actin was used as loading control. (c) MCF7 cells were treated for 48 h as indicated, or transfected with PPAR γ , FasL or control RNAs. Positions of procaspase 8 (P) and its active cleavage product (S) are indicated by arrowheads on the right. One of three similar experiments is presented. β -actin was used as loading control on the same stripped blot. (d) DNA laddering was performed in MCF7 cells treated for 72-h as indicated, or transfected with PPAR γ or control RNAs

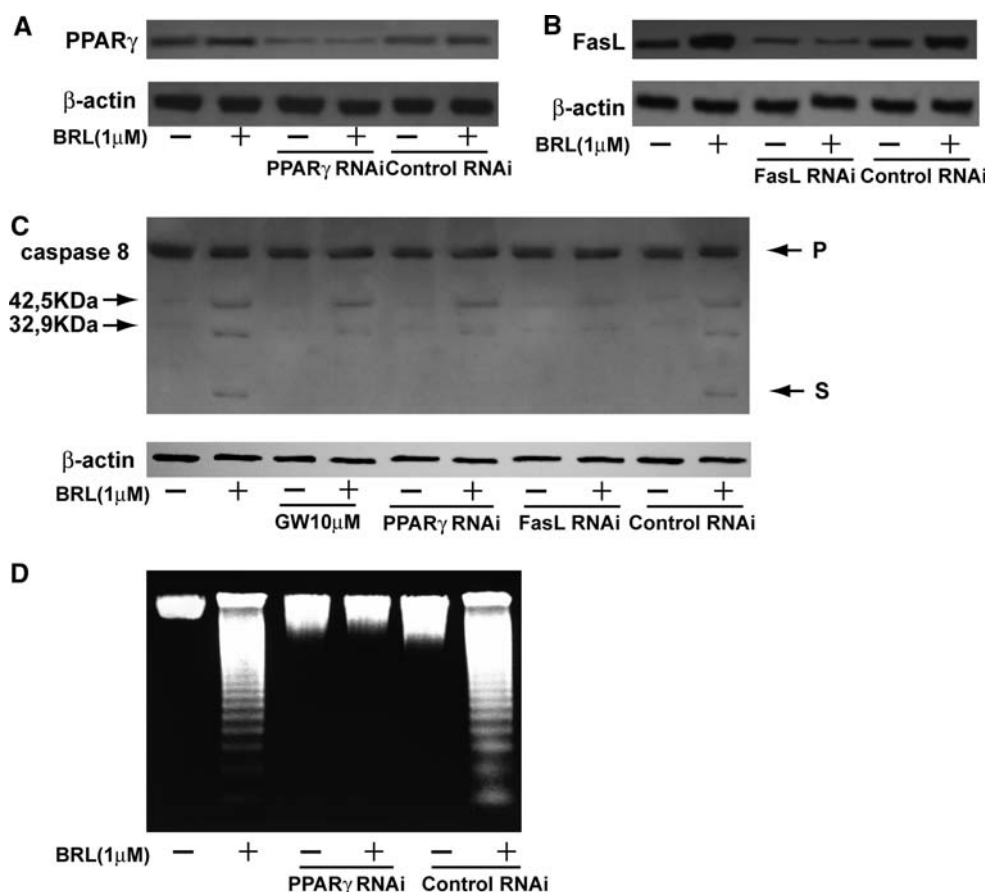
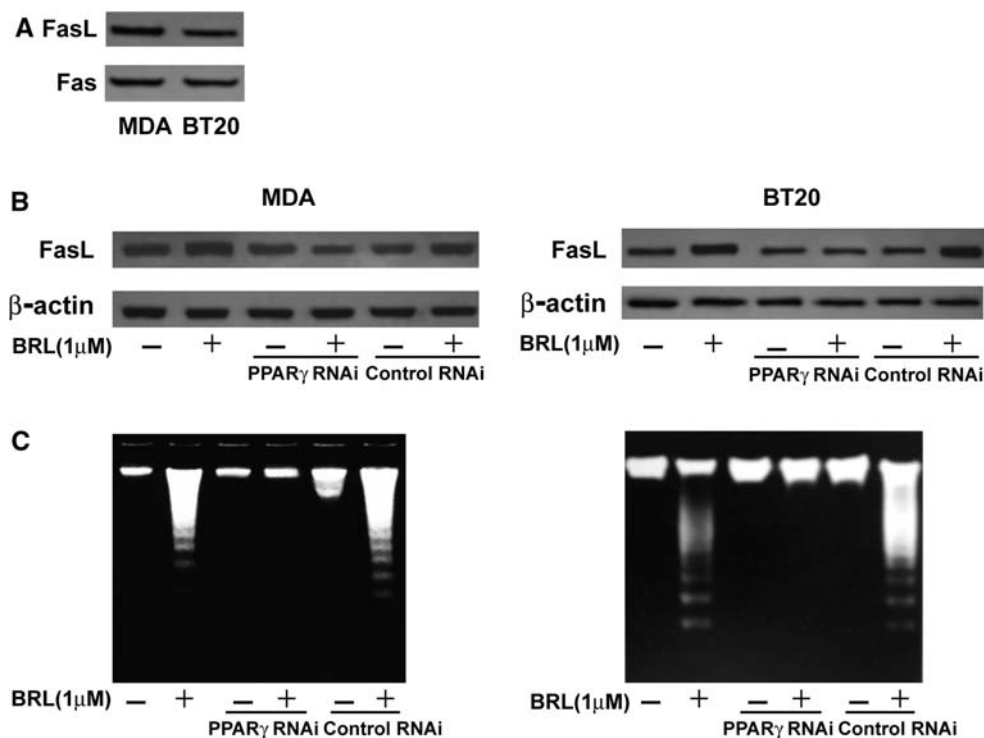


Fig. 6 BRL induces FasL up-regulation through PPAR γ in breast cancer cells. (a) FasL and Fas protein expression (evaluated by WB) in MDA and BT20 breast cancer cells. (b) MDA and BT20 cells were treated for 48 h as indicated, or transfected with a 25-nucleotide of RNA interference (RNAi) targeted human PPAR γ mRNA sequence or with control RNAi as reported in Materials and Methods. β -actin was used as loading control. One of three similar experiments is presented. (c) DNA laddering was performed in MDA and BT20 cells treated for 72-h as indicated, or transfected with PPAR γ or control RNAs



It has been described that breast cancer cells may be Fas sensitive or Fas insensitive and divergent data are presented on FasL expression in these cells [31, 32]. In our MCF7 cells, positive to Fas and FasL, we found that BRL through PPAR γ activation upregulated FasL at both protein and mRNA levels. Despite the FasL relevance, its cell and tissue distribution, and the apparent differences of its expression in a cell-specific manner, little knowledge is available how FasL expression is regulated. Recently, a downregulation of FasL by BRL has been reported in focal cerebral ischemia [44]. It was also showed that prostaglandin 15-deoxy- Δ 12,14-prostaglandin J2, a natural PPAR γ ligand inhibits FasL gene expression in T lymphocytes *via* a PPAR γ -independent mechanisms [45]. Indeed it is quite common for biological signalling pathways to vary depending on tissue type or cell condition.

We demonstrated that BRL increased the transactivation of FasL promoter in a dose-related and in a PPAR γ -dependent manner. Promoter deletion analysis of FasL has delineated a minimal promoter fragment spanning nucleotides from –318 to –237 bp, which was responsible for BRL activity. Indeed, the abovementioned region included three DNA motifs known to bind the transcription factors Sp1, NFAT and NF κ B [33]. NF κ B was reported to physically interact with PPAR γ [46], which in some circumstances binds to DNA cooperatively with NF κ B [26, 46, 47]. NFAT and Sp1 are critical factors responsible for FasL gene activation [48]. NFAT is a family of related transcription factors that plays a central role in regulating the immune response and it has been demonstrated the involvement of NFAT in FasL transcription [33, 49]. Several studies indicate that many mammalian gene types are controlled by Sp1, including genes for structural proteins, metabolic enzymes, cell cycle regulators, transcription factors, growth factors, surface receptors, and others [50]. PPAR γ has been documented to functionally interact with Sp1 to modulate gene expression [51, 52]. These findings are in agreement with our results since the PPAR γ -mediated transactivation of FasL promoter was abolished by using mithramycin and a construct deleted of the Sp1 site. By EMSA studies with nuclear extract from MCF7 cells, a binding of PPAR γ to the Sp1 sequence located within the FasL promoter was observed, it was enhanced by BRL and immunodepleted by both PPAR γ and Sp1 Abs, addressing the coexistence of the two proteins in the DNA binding complexes. In addition, the interaction between PPAR γ and FasL promoter was supported by ChIP assay where BRL treatment also increases the binding of RNA-Pol II to this promoter gene addressing a positive transcriptional regulation mediated by PPAR γ .

Engagement of the FasL homotrimer to three Fas molecules induces apoptosis by clustering of the receptor's death domains [14, 15]. This leads to the binding of FADD and caspase 8 to the receptor [14]. In the present study, we

obtained the active cleavage of caspase 8 under BRL treatment, that was no longer detectable in the presence of GW as well as inhibiting both the expression of FasL and PPAR γ by the respective RNAs. Previous works from our group have identified important pathways underlying the role of PPAR γ in cell growth, cycle arrest and apoptosis in MCF7 breast cancer cells [25, 26]. First, we evidenced an opposite effects of estrogen receptor (ER) α and PPAR γ on the regulation of the PI3K/AKT pathway, classically involved in cell survival and proliferation, eliciting divergent responses in presence of the cognate ligands 17 β -estradiol and BRL respectively in estrogen-dependent MCF7 cells [25]. Subsequently, we showed that BRL promotes the growth arrest and apoptosis in MCF7 cells through a crosstalk between p53 and PPAR γ . Our results indicated that in a consecutive series of events BRL: (1) up-regulates the expression of p53 and (2) its effector p21WAF1/Cip1, (3) triggers the cleavage of caspase 9, an important component of the intrinsic apoptotic pathway [26]. Finally, the DNA fragmentation assay clearly evidenced that PPAR γ was involved in the apoptotic process triggered by BRL since this effect was completely reversed by GW, while the apoptosis was only partially abolished inhibiting p53 expression by AS/p53, a plasmid encoding for p53 antisense [26]. The reported finding evidenced that the cross talk between PPAR γ and p53 is responsible, at least in part, in the BRL-induced apoptosis in MCF7 cells [26] and that alternative signalling cascades may be involved, like the Fas/FasL signalling that triggers the extrinsic apoptosis pathway, as we observed in this study. Interestingly, PPAR γ -induced apoptosis through FasL appears to be a general mechanism in breast cancer cells since it occurs in MCF7, MDA and BT20 cells.

In conclusion, we have shown that FasL expression is induced by PPAR γ through the binding to the transcription factor Sp1, demonstrating for the first time that PPAR γ triggers apoptotic events in breast cancer cells *via* Fas/FasL signalling pathway. However, the genomic response to PPAR γ activation remains complex and the understanding of the interactions between PPAR γ and other factors amplifies our knowledge on genes participating in cell-cycle modulation and apoptosis. The data presented place FasL as a novel molecular target to add to the variety of anticancer activities mediated by PPAR γ and further candidate PPAR γ -ligands for the treatment of patients with ER α + and ER α – breast cancer.

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